Saskatchewan
Ministry of
Agriculture

RESEARCH

FINAL REPORT

ADF

AGRICULTURE DEVELOPMENT FUND

20050731

OPTIMIZING FERTILITY AND OOCYTE QUALITY AFTER ARTIFICIAL INSEMINATION IN BEEF CATTLE

Funded by: The Agriculture Development Fund

April 2010

Prepared by: University of Saskatchewan (U of S)

OPTIMIZING OOCYTE QUALITY AND FERTILITY AFTER ARTIFICIAL INSEMINATION IN BEEF CATTLE

ADF Project No. 20050731
University of Saskatchewan Research Account Number 405600-1140-8000

Final Report

(a) **Abstract/Summary** - Maximum of one page in lay language. This must include project objectives, results, and conclusions for use in publications and in the Department database

The overall objective of the project was to investigate the effects of hormonal treatments designed to synchronize follicle growth and ovulation on oocyte maturation in beef cattle. Specifically, we tested the hypotheses that 1) A short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles), 2) Low circulating progesterone concentration (high LH pulse frequency) throughout the growing phase of the dominant follicle is detrimental to oocyte quality, and 3) A shortened duration of proestrus is associated with a decrease in fertility as a result of reduced oocyte quality. Studies involved the use of the University of Saskatchewan Goodale Farm and Agriculture and Agri-Food Canada beef herds to synchronize estrus and ovulation for fixed-time artificial insemination (AI) over the four-year period (approximately 400 cows). In addition, the effects of hormone treatments on oocyte and embryo quality were investigated through ovarian superstimulation and in vivo or in vitro fertilization. Ovarian ultrasound data, daily plasma hormone concentrations, and oocyte / embryo quality provided critical end-points for hypothesis testing.

Salient results were as follows:

- Long-duration of progesterone exposure during growth of the dominant follicle in lactating beef cows and beef heifers resulted in a larger diameter of the ovulatory follicle, but pregnancy rate was not significantly different than in the short-progesterone exposure group.
- Oocyte competence in superstimulated cows did not improve by shortening the duration of progesterone exposure during follicular growth. FSH starvation substantially reduced the ability of superstimulated follicles to ovulate.
- Low circulating progesterone concentrations during the growing phase of the ovulatory follicle resulted in a larger dominant follicle and a larger CL that produced more progesterone, with no significant effect on pregnancy rate.
- Higher doses of GnRH were more efficacious in inducing LH release in heifers, and exogenous estradiol increased LH release following treatment with GnRH. High concentrations of progesterone decreased both plasma LH concentrations and ovulation rates in heifers following treatment with GnRH.

- The progesterone environment (LH pulse-frequency) during the early growing phase after wave emergence did not influence oocyte competence in superstimulated heifers.
- The use of prostaglandin with or without exogenous progesterone treatment increased the percentage of heifers close to spontaneous puberty that ovulated following treatment.
- Shortening the period of proestrus resulted in dramatic decrease in pregnancy rate following fixed-time AI in beef cattle. A low progesterone environment during growth of the ovulatory follicle increased ovulatory follicle size and subsequent CL size and function, but it did not compensate for the effect of shortened proestrus on pregnancy rates.

Results of these studies were expected to increase fertility to AI in both beef and dairy cattle and help researchers to design effective, simplified and less expensive protocols for fixed-time artificial insemination. We thank the Saskatchewan Agricultural Development Fund for financially supporting the operational costs of this project. It is noteworthy that we were also supported by a cash contribution of $15,000 from Westgen Inc. and the veterinary pharmaceutical industry contributed the hormones needed for this project (in-kind contribution of more than $50,000).

(b) **Introduction** - Brief project background, rationale and objectives

Highly efficacious estrus synchronization protocols that permit artificial insemination (AI) without the need for estrus detection have been developed. Now that we know how to control ovarian function in the cow, we can turn our attention to the developmental capability of the ovulated egg; i.e., increase fertility. Other than pregnancy rates subsequent to synchronization, little attention has been paid to the effects of hormonal treatments on the developmental capacity of the oocyte within the ovulatory follicle of the synchronized wave. **The overall objective of this project was to determine the effects of hormonal treatments on oocyte competence, and ultimately on fertility, following fixed-time AI**. Specifically, we investigated the following endocrine effects on oocyte quality and fertility:

1) Duration of progesterone influence during development of the ovulatory follicle.
2) Low progesterone concentrations (which results in high LH levels) during the entire growing phase of the ovulatory wave.
3) Duration of the period of proestrus (luteolysis to ovulation).

Briefly, follicle growth in the cow occurs in a wave-like fashion (Pierson and Ginther, 1987, 1988; Savio et al., 1988; Ginther et al., 1989a; Ginther et al., 1989c; Ginther et al., 1989b; Knopf et al., 1989). The estrous cycle in cattle is composed of either 2 or 3 follicular waves, comprised of a group of growing antral follicles from which a dominant follicle is selected; the remaining follicles become subordinates and undergo atresia. In both 2- and 3-wave estrous cycles, the first follicular wave emerges on the day of ovulation (Day 0) while the second wave emerges on Days 9 or 10 in 2-wave cycles, and on Days 8 or 9 in 3-wave cycles (Adams, 1999). The third wave emerges on Days 15 or

16 in 3-wave cycles. The dominant follicle present at the time of luteolysis will become the ovulatory follicle. The duration of the estrous cycle is 20 days in 2-wave cycles and 23 days in 3-wave cycles (Adams, 1999). The proportion of animals with 2- and 3-wave cycles is approximately equal. The fundamental information about the dynamics of follicular waves created a flurry of interest and led to a series of advancements in our understanding of folliculogenesis and the development of methods to control ovarian function in cattle and other species (Adams 1994, 1998, 1999; Adams et al, 2008; Fortune 1993, Ginther et al., 1996, 2001a)

Recruitment of follicular waves and selection of a dominant follicle is based on differential responsiveness to follicle stimulating hormone (FSH) and luteinizing hormone (LH). Surges in plasma FSH elicit the emergence of a follicular wave (Adams et al., 1992b); FSH is subsequently suppressed by products of the growing follicles. Therefore, FSH is declining at the time when the growth profiles of the dominant and subordinate follicles begin to diverge (time of selection), 2 to 3 days after wave emergence (Adams et al., 1992a; Adams et al., 1993). Within each wave, the follicle that first acquires LH receptors becomes the dominant follicle (Beg et al., 2001; Ginther et al., 2001b), whereas subordinates, that still require FSH, undergo atresia. The dominant follicle grows for approximately 6 days and then enters a static phase. Suppression of LH as a consequence of progesterone secretion by the corpus luteum (CL) causes the dominant follicle to cease its metabolic functions after 2 or 3 days; thereafter, it begins to regress and FSH surges again (Adams, 1999; Ginther et al., 2001a). The FSH surge is responsible for eliciting the emergence of the next wave (Adams et al., 1992b). The ovarian cycle then repeats itself. Luteal regression allows LH pulse frequency to increase, which in turn promotes the growth of the dominant follicle and the production of increasing amounts of estradiol (Ginther et al., 2000a, 2000b). Once estradiol concentrations reach a threshold, a positive feed-back effect on the hypothalamo-pituitary axis results in a surge of LH, followed by ovulation.

A clear understanding of the endogenous control of follicle growth and ovulation in the cow has resulted in the development of tools to control these events for the purposes of advanced reproductive technologies, including fixed-time AI (Bo et al., 1995; Mapletoft et al., 1999; Bo et al., 2002; Mapletoft et al., 2002) and prediction of the superstimulatory response (Singh et al., 2004). Progesterone is a key hormone controlling many of these events; it suppresses LH release (Rawlings et al., 1984) and causes LH-dependent follicles to regress, whereas estradiol suppresses FSH release and causes FSH-dependent follicles to regress (Adams, 1999). Removal of estradiol-secreting follicles (ablation, induction of ovulation or by estradiol treatment) results in FSH release and emergence of a new wave (Bo et al., 1993; Bergfelt et al., 1994; Bergfelt et al., 1997; Bo et al., 2002). Injection of LH, GnRH or estradiol can result in an LH release and synchronous ovulation of an LH-dependent follicle. The utilization of this knowledge has provided the means for fixed-timed AI, without the necessity of estrus detection. However, this knowledge should also provide the basis by which fertility can be improved, especially as we increase our understanding of oocyte development, fertilization, and early embryo development. This project was aimed at investigating the role of progesterone in conferring oocyte competence, and consequently fertility. Rationale for specific

hypotheses are discussed in the Methods and Result section.

(c & d) **Methods and Results**- Include approaches, experimental design, methodology, materials, sites, etc. Major changes from original plan should be indicated and the reason(s) for the change should be specified. Present and discuss project results, including data, graphs, models, maps, design and technology development.

Objective 1

Effect of progesterone during the growing phase of the ovulatory follicular wave on oocyte quality and fertility.

Rationale:
Now that we are able to control ovarian function in cattle for fixed-time AI, we can focus on methods of improving fertility. Other than assessing pregnancy rates, little attention has been given to the effects of hormone treatments on the competence of the oocyte present in the ovulatory follicle of the synchronized estrus. Therefore, studies were proposed to investigate the effects of specific hormonal treatments (for the synchronization of follicle growth and ovulation) on oocyte quality and fertility. As progesterone plays such an important role in the secretion of LH, these studies were directed toward optimizing oocyte competence by manipulating circulating progesterone concentrations. It has been reported that fertility is higher in cows with three follicular waves than in those with two follicular waves. The interval from wave emergence to ovulation in 2-wave cycles is about 9 days, but only 6 days in 3-wave cycles. The ovulatory waves differ between 2- and 3-wave cycles in the duration of progesterone exposure at the beginning of the wave (i.e., 6 days vs. 3 days, respectively) while the progesterone-free period is similar (3 days).

Hypothesis:
A short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles).

Study 1: Effect of duration of progesterone exposure during development of the ovulatory follicle on pregnancy rate in cattle

The objective was to determine the effect of duration of progesterone exposure during the ovulatory wave on pregnancy rates in beef cattle. We tested the hypothesis that a short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles).Three to 5 d after ovulation, beef heifers (n=172) and suckled beef cows (n=193) received an intravaginal insert containing 1.9 g progesterone (CIDR) and 2.5 mg estradiol-17β + 50 mg progesterone im to induce a new follicular wave. Cattle were allocated to short- or long-progesterone exposure groups (for 3 and 6 d after wave emergence, respectively), analogous to the ovulatory wave of 3- and 2-wave

estrous cycles. At the end of progesterone treatment, prostaglandin F2α was administered and CIDR inserts were removed. Forty-eight hours later, all cattle were given 12.5 mg pLH, and concurrently artificially inseminated with frozen-thawed semen. Diameter of the two largest follicles and the CL were measured by transrectal ultrasonography at CIDR removal, insemination, and 36 h after AI. Ultrasonographic pregnancy diagnosis was done 38 and 65 d after AI.

Table1. Pregnancy rates in cows and heifers exposed to a short or long-progesterone environment during development of the ovulatory wave.

Pregnancy rate	**Short-progesterone**	**Long-progesterone**
Cows	59/94 (63%)	58/99 (58%)
Heifers	45/85 (53%)	41/87 (47%)
Total	104/179 (58%)	99/186 (53%)

There was no significant difference in pregnancy rates following short- vs. long-duration progesterone exposure in heifers (Table 1). However, the diameter of the ovulatory follicle at CIDR removal and AI was smaller in short- than in long-duration groups ($P<0.02$), and larger in cows than in heifers ($P<0.006$). In conclusion, although long-duration progesterone exposure during growth of the ovulatory follicle resulted in a larger diameter on the last examination before ovulation, pregnancy rate was not significantly different than in the short-progesterone exposure group.

This work was presented at the following conferences and has been submitted for publication. Please see the appended manuscript for details.

Dias FCF, Stover BC, Mapletoft RJ, Kastelic J, Adams GP, Colazo M, Dochi O, Singh J (2010) Duration of progesterone exposure during development of the ovulatory follicle does not affect pregnancy rate in cattle (Manuscript).

Dias FCF, Stover BC, Mapletoft RJ, Kastelic J, Adams GP, Colazo M, Dochi O, Singh J (2007) Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate *Proceedings of the 53rd Annual Meeting of the Canadian Fertility and Andrology Society* Halifax NS Canada September 26-29, 2007 Abstract#TP16 (poster).

Dias FCF, Stover BC, Mapletoft RJ, Kastelic J, Adams GP, Colazo M, Dochi O and Singh J (2007) Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate in cattle *Reproductive Science and Medicine Research Symposium* (March 9, 2007) and *14th Life sciences and Health Sciences Research Conference* Saskatoon Saskatchewan Canada March 16, 2007 (oral presentation and poster)

Stover B, Dias FC, Mapletoft RJ, Kastelic J, Adams GP and Singh J (2006) Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate *WCVM Poster Day* Saskatoon Saskatchewan Canada September 06-08, 2006 (poster).

Study 2: Effect of length of progesterone and growing phase of the ovulatory follicles on oocyte competence after superstimulatory treatment

The objective was to determine the effect of duration of progesterone exposure, during the growing phase of ovulatory follicles, on oocyte competence in superstimulated cows. We tested the hypotheses that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth, but is not affected by FSH starvation at the end of superstimulatory treatment. Forty cross-bred beef cows (weight, 515 to 795 kg) were used. Transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter was done 5 to 8 days after ovulation, and a progesterone-releasing device (CIDR) was placed intravaginally. Cows were allocated randomly to three groups; those in the short P_4 and FSH starvation groups received 8 injections of FSH (each was equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals, whereas the long P_4 group received 14 injections of FSH. In all groups, FSH treatment started on the day of wave emergence (Day 0) approximately 1 d after follicle ablation. Cows in the short P_4 group were given 25 mg of PGF twice, (12-hours apart), on Day 3, whereas cows in the two other groups received two injections of PGF on Day 6. In all cows, CIDR were removed at the time of second PGF treatment; 25 mg pLH was given im 24 hours after CIDR removal and cows were inseminated 24 and 36 hours later. Reproductive tracts were collected at slaughter, 4 days after insemination and ova/embryos were evaluated and cultured for 10 days to determine developmental competence. Data from cows that failed to ovulate (6 of 13 and 1 of 13 in the FSH starvation and long P_4 groups, respectively), were excluded from the analyses.

Table 2 The effect of duration of progesterone exposure and FSH starvation on the mean (± SEM) number of follicles during superstimulatory treatments in cows.

	Short P_4	FSH starvation	Long P_4	P value
No. follicles	n = 14	n = 13	n = 13	-
8th dose of FSH				
≥3 mm	18.6 ± 2.5	19.2 ± 1.8	25.4 ± 2.1	0.06
≥7 mm	10.5 ± 2.0	5.7 ± 1.2	9.6 ± 1.5	0.10
CIDR removal				
≥3 mm	18.6 ± 2.5	22.5 ± 2.1	25.3 ± 2.5	0.14
≥7 mm	10.5 ± 2.0[a]	9.1 ± 1.8[a]	18.1 ± 2.7[b]	0.02
12 hours after second AI				
≥3 mm	15.8 ± 4.2	15.8 ± 3.5	13.33 ± 2.3	0.88
≥7 mm	5.4 ± 1.6	9.0 ± 2.1	5.2 ± 1.5	0.20
Estimated number (%) of ovulations between CIDR removal and 12 hours after second AI	5.1 (71.3%)[ab]	0.1 (1.1%)[a]	12.9 (48.6%)[b]	0.02

[a,b]- Within a row, groups without a common superscript differed (P<0.05)

Table 3. Mean (±SEM) numbers of ovulations and ova/embryos recovered at slaughter 4 days after insemination in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment.

	Short P_4	FSH starvation	Long P_4	P value
No. cows	14	13	13	-
No. ovulations	11.6 ± 2.2^a	2.5 ± 1.2^b	15.4 ± 3.0^a	0.001
Total ova/embryos recovered	5.9 ± 1.3^a	0.9 ± 0.6^b	6.7 ± 1.5^a	0.048
Recovery rate (%)	50.9^a	36.0^b	43.5^a	0.048
No. uncleaved ova (%)	13/82 (15.9)	1/12 (8.3)	5/87 (5.7)	0.09
No. cleaved ova (%)	69/82 (84.1)	11/12 (91.7)	82/87 (94.2)	0.09
Grades 1 or 2 embryos (%)	87.0^a	81.8^a	37.8^b	0.0001
Grades 3 or 4 embryos (%)	13.0^a	18.2^a	62.2^b	0.0001

[a,b]- Within a row, values without a common superscript differed ($P<0.05$)

When compared to the short P_4 or long P_4 groups, the FSH starvation group had fewer CL (i.e. ovulations; Table 3) at slaughter. However, the proportion of Grades 1 and 2 embryos 4 days after insemination was higher in the short P_4 and FSH starvation groups than in the long progesterone group (60 of 69, 9 of 11, and 31 of 82, respectively; $P<0.001$), with no significant difference in embryo quality between long and short P_4 groups 7, 9, and 10 days after AI (there were too few embryos in the FSH starvation group to evaluate). In conclusion, the hypothesis that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth was not supported, whereas the hypothesis that oocyte competence is not affected by FSH starvation at the end of superstimulatory treatment was not adequately tested. Notwithstanding, FSH starvation substantially reduced the ability of superstimulated follicles to ovulate.

This work was presented at the following conferences and has been submitted for publication. Please see the appended manuscript for details.

Dias FCF, Costa E, Adams GP, Mapletoft RJ, Dochi O, Singh J (2010) Effect of length of progesterone exposure during the growing phase of the ovulatory follicular wave on oocyte competence after superovulatory treatment (Manuscript)

Dias FCF, Costa E, Adams GP, Mapletoft RJ, Dochi O, Singh J (2008) Effect of length of progesterone exposure during the growing phase of the ovulatory follicular wave on oocyte competence after superovulatory treatment *Proceedings of the 16th International Congress on Animal Reproduction* Budapest Hungary, July 2008. *Reproduction in Domestic Animals* **43** 37 (Abstract # P029)

Objective 2

Effect of low versus high progesterone concentrations, during ovulatory follicle growth, on oocyte quality and fertility.

Rationale:
There are two emerging hypotheses concerning gonadotropin requirements for oocyte maturation: firstly, follicles (and oocytes) require high LH pulse frequency prior to the ovulation-inducing LH surge, and secondly, follicles (and oocytes) require a period of low FSH before the final phase of maturation. Our results from previous studies (Nasser et al., 1993) demonstrated that the ovarian response was similar when superovulatory treatment was initiated during the first or the second waves. However, more recent results suggested that although the ovarian response is similar, embryo production (fertility) is significantly lower from first-wave than second-wave ovulations. A fundamental difference in the hormonal milieu of the 2 waves is that the growing dominant follicle of Wave 1 is exposed to much lower concentrations of progesterone than its Wave 2 counterpart.

Hypothesis:

Low circulating progesterone concentration (high LH pulse frequency) throughout the growing phase of the dominant follicle is detrimental to oocyte quality.

Study 3: Effects of low versus physiologic (high) plasma progesterone concentrations on ovarian follicular development and fertility in beef cattle

The objective of this study was to determine the effects of low versus physiologic plasma progesterone concentrations during the ovulatory wave on fertility in cattle. Suckled beef cows (Bos taurus; n=129) and pubertal heifers (Bos taurus; n=150) at random stages of the estrous cycle were given a luteolytic dose of prostaglandin F2α (500 microg cloprostenol; PGF) twice, 11 d apart. Ten days after the second PGF treatment, cattle were given estradiol benzoate im (1.5 and 1.0 mg for cows and heifers, respectively) and a progesterone-releasing intravaginal device (Cue-Mate) with a single pod containing 0.78 g progesterone (Day 0). Cattle in the low-progesterone group (n = 148) received a luteolytic dose of PGF on Day 0, whereas those in the high-progesterone (i.e., physiologic plasma concentrations) group (n=131) were allowed to retain their corpora lutea. On Day 8, the Cue-Mates were removed, and PGF was given to both groups. Fifty-four to 56 hours later, cattle received 12.5 mg of porcine LH (pLH; Lutopin-V) im and were concurrently artificially inseminated. The dominant follicle in the low-progesterone group was larger ($P<0.001$) than that in the high-progesterone group on the day of insemination (14.9 ± 0.3mm vs. 12.7 ± 0.3mm, mean$\pm$SEM). At 7 d after ovulation, the low-progesterone group had a larger corpus luteum (24.5 ± 0.54 mm vs. 21.9 ± 0.64 mm, $P<0.01$) and higher plasma progesterone concentration (4.0 ± 0.3 vs. 3.1 ± 0.2, $P<0.01$) than that of the high-progesterone group. However, pregnancy rates did not differ (79 of 148, 53.4%, and 70 of 131, 53.4%) for low- and high-progesterone groups, respectively). Results are shown in Table 4.

Table 4. Reproductive end points in cows and heifers (category) assigned to treatment with low- (LP) or high- (HP) progesterone (treatment).

	Heifer		Cow		P-Value**		
	LP	HP	LP	HP	Trt	Cat	Trt*Cat
Pregnancy rate (%)*	55 (44/80)	50 (35/70)	51.5 (35/68)	57.4 (35/61)	0.9	0.81	0.37
Ovulation rate (%)*	83.8 (67/80)	77.1 (54/70)	81.4 (55/68)	84.0 (51/61)	0.64	0.76	0.33
Diameter of DF at Cue-Mate removal (mm)	12.2± 0.34^{aA}	10.2± 0.55^{bB}	14.3± 0.51^{aC}	12.3± 0.34^{bA}	0.002	0.07	0.93
Diameter of DF at AI (mm)	13.6± 0.35^{aA}	11.5± 0.48^{bB}	16.5± 0.52^{aC}	14.2± 0.34^{bA}	0.001	0.001	0.76
CL diameter 7 d after AI (mm)	24.2± 0.83^{aA}	20.8± 1.06^{bB}	24.7± 0.69^{aA}	22.2± 0.76^{bAB}	0.004	0.3	0.64

[b]Values without a common superscript differed between treatments within a category.
[A,B]Values without a common superscript differed between categories.
* Pregnancy rate and ovulation data were analyzed by logistic regression; the remaining data were analyzed by two-factor ANOVA.
**P-values from statistical analyses for treatment (Trt; LP vs HP), category (Cat; cows vs heifers) and treatment x category interaction (Trt*Cat) are provided in last three columns.

In summary, low circulating progesterone concentrations during the growing phase of the ovulatory follicle resulted in a larger dominant follicle and a larger CL that produced more progesterone, with no significant effect on pregnancy rate.

This work was presented at the following conferences and has been published in Theriogenology. Please see the appended article for details.

Pfeifer LF, Mapletoft RJ, Kastelic JP, Small JA, Adams GP, Dionello NJ, Singh J. (2009) Effects of low versus physiologic plasma progesterone concentrations on ovarian follicular development and fertility in beef cattle *Theriogenology* 72 1237-50.

Pfeifer LF, Mapletoft R, Adams G, Kastelic J, Small J, Dias F, Singh J (2008) The effect of progesterone on follicular development and pregnancy rate in beef cattle *Reproductive Science and Medicine Program Symposium* University of Saskatchewan Saskatoon Canada February 29, 2008 (oral presentation).

Pfeifer LF, Mapletoft R, Adams G, Kastelic J, Small J, Dias F, Singh J (2008) The effect of progesterone on follicular development and pregnancy rate in beef cattle *Proceedings of the 34th annual conference of the International Embryo Transfer*

Society Denver Colorado USA January 5-9, 2008 Abstract # 2345. *Reproduction Fertility and Development* 20 p90 (poster)

Study 4: Effect of circulating concentrations of progesterone on the developmental competence of oocytes in superovulated cows

The study was designed to determine the effect of progesterone (and hence, LH pulsatility) during superstimulatory treatment on the competence of the oocyte to develop into an embryo. The growing phase immediately after wave emergence (Day 0) was subjected to a relatively high, low, or prolonged-low progesterone environment (n=10/group) through the use of progesterone-releasing intravaginal device (CIDR) and luteolytic dose of PGF. Heifers were given 200 mg FSH divided bid over 4 days beginning on Day 0. The CIDR were removed from low- and high-progesterone groups on Day 3, and from the prolonged-low-progesterone group on Day 8. Heifers were given pLH 24 hours after CIDR removal, inseminated artificially at 24 and 36 hours after pLH, and slaughtered 3 days after AI. Plasma progesterone was elevated in the high-progesterone group ($P<0.01$), and plasma LH pulse frequency was elevated in low- and prolonged-low-progesterone groups ($P<0.01$). Multiple follicles developed in all groups, but failed to ovulate in the prolonged-low-progesterone group. A higher total recovery rate in the high- vs. low-progesterone group (68% vs. 50%, $P<0.01$) was associated with the collection of a greater number of unfertilized oocytes. However, no differences between the high- and low-progesterone groups were detected in the number of ovulations or in the number of embryos that developed to the 2- to 16-cell stages. We conclude that the progesterone environment (LH pulse-frequency) during the early growing phase after wave emergence does not influence oocyte competence in superstimulated heifers. Results provide rationale for the hypothesis that prolonged exposure to high LH, without continued FSH support, permits continued follicular growth but a loss of ovulatory capability. Results are shown in Table 5.

Table 5. Comparison of response to the superstimulatory treatment and rate of ova/embryo transport in the genital tract of heifers under low (n = 10) vs. high-progesterone (n = 10) environments.

End-points		Low progesterone	High progesterone
Ovulations per heifer (CL detected at slaughter; mean ± SEM)		22.3 ± 4.1[a]	20.4 ± 4.7[a]
Total oocytes and embryos collected per ovulation		111/223 (50%)[a]	139/204 (68%)[b]
Unfertilized oocytes collected per ovulation		7/223 (3%)[a]	37/204 (18%)[b]
Embryos collected per ovulation		104/223 (47%)[a]	102/204 (50%)[a]
2-cell embryos		0/104 (0%)[a]	2/102 (2%)[a]
	(mean ± SEM)	0[a]	0.2 ± 0.1[a]
4-cell embryos		8/104 (8%)[a]	13/102 (13%)[a]
	(mean ± SEM)	0.8 ± 0.5[a]	1.3 ±0.6[a]
8-cell embryos		55/104 (53%)[a]	58/102 (57%)[a]
	(mean ± SEM)	5.5 ±1.1[a]	5.8 ± 2.3[a]
16-cell embryos		41/104 (39%)[a]	29/102 (28%)[a]
	(mean ± SEM)	4.1 ± 1.4[a]	2.9 ± 1.9[a]

Oocytes + embryos recovered from uterine tubes	17/111 (15%)[a]	51/139 (37%)[b]

[ab]Values within rows with no common superscript are different ($P < 0.05$)

This work was presented at the following conferences and has been submitted for publication. Please see the appended manuscript for details.

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP (2009) Oocyte competence under different progestational environments (manuscript)

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP (2006) Oocyte competence under different progestational environments *Proceedings of the 39th Annual Meeting of the Society for the Study of Reproduction* Omaha Nebraska USA July 29-August 1, 2006. Biology of Reproduction 2006 Special Issue p109, Abstr. # 166.

Study 5: Effect of progesterone and estradiol on GnRH-mediated LH secretion

We hypothesized that increased doses of GnRH or exogenous estradiol (E_2) will enhance GnRH-induced LH release in heifers with elevated plasma progesterone (P_4) concentrations. Two experiments (both 2 x 2 factorial designs) were conducted using nulliparous, postpubertal Angus-cross heifers. In Experiment 1, all heifers (n=32) had their two largest ovarian follicles ablated 5 d after ovulation, and they were given a once-used CIDR. Furthermore, these heifers were randomly assigned to be given either PGF (immediately and 12 h later; Low-P_4) or no treatment (High-P_4). Six days after emergence of a new ovarian follicular wave (detected by ultrasonography), half of the heifers in each group (n=8) were given either 100 or 200 µg of GnRH (Cystorelin). There were significant effects of progesterone, GnRH dose, time, and their interactions, on plasma LH concentrations after GnRH treatment; plasma LH concentrations were higher in the Low-P_4 than in the High-P_4 group, and in heifers given 200 vs 100 µg of GnRH (15.4 vs 9.1 and 14.7 vs 9.8 ng/mL, respectively). Ovulation rate was higher ($P=0.002$) in the Low-P4 group (15/16) than in the High-P4 group (6/16), but GnRH dose had no effect ($P=0.4$). In Experiment 2, heifers (n=22) were treated similarly, except that 5.5 d after wave emergence, half the heifers in each group were further divided to be given 0.25 mg estradiol benzoate im or nothing, and 8 h later, all heifers were given 100 µg GnRH. The two groups that were given E_2 (Low- and High-P_4) and the Low-P_4 group without E_2 had higher peak plasma LH concentrations ($P<0.04$) compared to the group with high progesterone without E_2 (12.6±1.8, 10.4±1.8, 8.7±1.3, and 3.9±1.2 ng/mL). However, E_2 did not affect ovulation rates ($P=0.6$). Combined for both experiments, heifers with low plasma progesterone concentrations had higher LH concentrations following GnRH treatment (11.5 vs 6.4 ng/mL, $P=0.039$). In conclusion, the hypotheses that higher doses of GnRH will be more efficacious in inducing LH release and that exogenous estradiol will increase LH release following treatment with GnRH were supported. Moreover, high concentrations of progesterone decreased both plasma LH concentrations and ovulation rates. Results are shown in Figure 1.

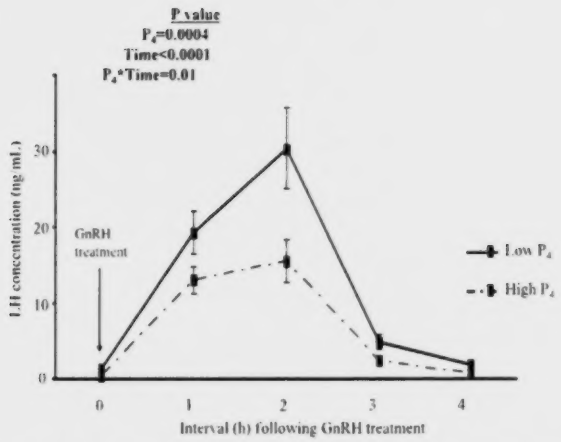


Figure 1. Mean (±SEM) plasma concentrations of LH in response to treatment with 100 µg GnRH (Cystorelin) in heifers with low versus high plasma concentrations of progesterone (P_4; n=11 per group, combined for Experiments 1 and 2).

This work have been submitted to Domestic Animal Endocrinology for publication. Please see the appended manuscript for details.

Dias FCF, Colazo MG, Kastelic JP, Mapletoft RJ, Adams GP, Singh J (2009) Progesterone concentration, estradiol pre-treatment, and dose of GnRH affect GnRH-mediated LH release in beef heifers. (Manuscript)

Study 6: Effects of exogenous progesterone and cloprostenol on ovarian follicular development and first ovulation in prepubertal heifers

The objective of this study was to determine the effects of progesterone and cloprostenol (a PGF2α analogue) on ovarian follicular development and ovulation in prepubertal heifers. In Experiment 1, crossbred Hereford heifers (Bos taurus; 10 to 12 mo old, 255 to 320 kg) that had follicular activity but had not yet had their first ovulation (prepubertal) were assigned randomly to three groups and given (1) an intravaginal progesterone-releasing insert (CIDR; P_4 group, n=13); (2) a CIDR plus 500 µg cloprostenol im (PGF analogue) at CIDR removal (P_4PG group, n=11); or (3) no treatment (control group, n=14). The CIDR inserts were removed 5 d after follicular wave emergence. Progesterone-treated heifers (P_4 and P_4PG groups) had a larger dominant follicle than that of the control group (P=0.01). The percentage ovulating was highest in the P_4PG group (8 of 11, 73%), intermediate in the P_4 group (4 of 13, 31%), and lowest in the control group (1 of 14, 7%; P<0.02). In Experiment 2, 16 prepubertal heifers (14 to 16 mo old, 300 to 330 kg) were designated to have follicular wave emergence synchronized with either a CIDR and 1 mg estradiol benzoate im (EP group, n=8) on Day 0 (beginning of experiment) or by transvaginal ultrasound-guided ablation of all follicles ≥5 mm on Day 3 (FA group, n=8). On Day 7, CIDRs were removed in the EP group, and all heifers received 500 µg cloprostenol im. Ovulation was detected in 6 of 8 heifers (75%) in both

groups. In summary, the use of PGF2α with or without exogenous progesterone treatment increased the percentage of heifers close to spontaneous puberty that ovulated following treatment (Table 6).

Table 6. Rates of ovulation and proestrous signs and mean (±SEM) characteristics of the follicular wave of prepubertal heifers treated with a CIDR (P_4 group), a CIDR plus $PGF_{2\alpha}$ (P_4PG group), or no treatment (Control group) in Experiment 1.

	P_4 group	P_4PG group	Control Group	P-value
Ovulation rate	4/13 (30.8%)[a]	8/11 (72.7%)[a]	1/14 (7.1%)[b]	<0.001 *
Proestrus signs	8/13 (61.5%)[a]	9/11 (81.8%)[a]	2/14 (14.3%)[b]	<0.002 *
Interval from wave emergence to ovulation (d)	8.0±0.0[a]	8.5±0.25[ab]	10.0[b]	0.03
Maximum diameter of dominant ovulatory follicle (mm)	12.5±0.25[a]	12.7±0.34[a]	11.5±0.25[b]	<0.01
Maximum diameter of dominant anovulatory follicle (mm)	12.5±0.26[a]	11.3±0.16[b]	11.3±0.29[b]	0.017
Growth rate of all dominant follicles (mm/d)	1.1±0.04[a]	1.1±0.06[a]	0.9±0.02[b]	<0.03

Different superscripts in a row indicate a significant difference ($P<0.05$)

This work was presented at the following conferences and has been published in Theriogenology. Please see the appended article for details.

Pfeifer LF, Siqueira LG, Mapletoft RJ, Kastelic JP, Adams GP, Colazo MG, Singh J (2009) Effects of exogenous progesterone and cloprostenol on ovarian follicular development and first ovulation in prepubertal heifers *Theriogenology* **72** 1054-1064.

Pfeifer LF, Siqueira LG, Mapletoft RJ, Adams GP, Singh J (2008) Effect of exogenous progesterone on follicular development and ovulation rate in prepubertal heifers *Proceedings of Annual Conference of SBTE (Brazilian Embryo Transfer Society)* Guarujá Brazil. August 21-24, 2008. Acta Scientiae Veterinariae 30: 457 (poster).

Chaîné K, Pfeifer LFM, Mapletoft RJ, Kastelic J, Colazo M and Singh J (2007) Ovulation induction in peri-pubertal heifers: Follicular dynamics and endocrine changes *The 2007 Merck-Merial Symposium* National Institutes of Health Bethesda USA August 02-04, 2007 and *WCVM Poster Day* University of Saskatchewan Saskatoon Canada September 6, 2007 (poster).

Objective 3

Effect of duration of preovulatory maturation phase on oocyte quality and fertility.

Rationale:
There is increasing evidence that the length of proestrus (i.e., interval between luteal regression and ovulation) affects the function of the subsequent CL. However, there is no information on the effect of the duration of proestrus on oocyte quality. Fertilization rates in superstimulated cattle are lower than unstimulated (single-ovulation) cattle and we suspect that the discrepancy between these 2 groups in the interval between luteal regression and ovulation (approximately 72 versus 96 hr) may be the cause.

Hypothesis:
A shortened duration of proestrus is associated with a decrease in fertility as a result of reduced oocyte quality.

Study 7: Effect of short- versus long-proestrus on fertility in beef cattle after fixed-time artificial insemination

Selection and early growth of an ovulatory dominant follicle normally occurs in an environment of high-progesterone, while its final stages of growth (proestrus) occur in a low-progesterone environment. We tested the hypotheses that a) luteal-phase plasma progesterone exposure to growing dominant follicle followed by short duration of proestrus will result in lower fertility than longer proestrus, and b) low progesterone exposure during early growth of dominant follicle will overcome the effects of short proestrus. Heifers (n=61) and postpartum beef cows (n=80) were assigned randomly to four groups: high progesterone-long proestrus (HL), high progesterone-short proestrus (HS), low progesterone-long proestrus (LL) and low progesterone-short proestrus (LS). Cattle received two doses of PGF, 11 d apart, to synchronize the estrous cycle. Nine days later (Day 0), estradiol-17β and progesterone were given im to synchronize follicular wave emergence and a once-used CIDR device was inserted intravaginally to maintain low plasma progesterone concentrations. At the same time, low progesterone groups (LL & LS) were given two doses of PGF 12 h apart, while, high progesterone groups (HL & HS) were allowed to retain their CL. The CIDR were removed on Day 7 (HL, LL) or Day 8 (HS, LS). All animals received PGF on the day of CIDR removal and pLH 12 h later to induce ovulation. Artificial insemination was done 12 h after the pLH injection. Transrectal ultrasound was used to measure the follicular diameter on the day of AI, CL diameter on Day 9.5 post-AI, and for pregnancy diagnosis 60 d post-AI. Follicle and CL diameter data were analyzed by ANOVA and pregnancy rates were compared by the logistic regression. Low progesterone and long proestrus resulted in higher pregnancy rates ($P<0.023$; 54.0%, 38.2%, 46.4% and 12.2%, in LL, LS, HL and HS groups, respectively). There was no interaction between levels of progesterone and duration of proestrus ($P=0.12$) and the pattern was similar in cows and heifers ($P=0.47$). The size of the preovulatory follicle was larger ($P=0.001$) in low progesterone groups than in high progesterone groups, and in cows than in heifers ($P=0.039$). The CL diameters on Day 9 post-AI were smaller and progesterone concentrations were lower in high-progesterone groups ($P=0.023$, $P=0.003$, respectively) and in the short-proestrus

groups (P=0.003, P=0.0002, respectively) than in low-progesterone groups and long-proestrus groups. In summary, a short proestrous interval resulted in a reduced pregnancy rate following fixed-time AI in beef cattle. Although a low progesterone environment during growth of the ovulatory follicle increased ovulatory follicle size and subsequent CL size and function, it did not compensate for the effect of shortened proestrus on pregnancy rates.

This work won the student award at 2009 Annual Convention of American College of Theriogenology and the Society for Theriogenology, Albuquerque, New Mexico USA and was presented at the following conferences. Manuscript is under preparation.

Dadarwal D, Mapletoft RJ, Adams GP, Creelman C, Singh J (2009) The effects of progesterone concentration and duration of proestrus on fertility in beef cattle after fixed-time artificial insemination *Proceedings of the Reproductive Science and Medicine Research Symposium* February 27, 2009 (page 7, oral presentation) and *Life Sciences Day* University of Saskatchewan Saskatoon Canada

Dadarwal D, Mapletoft RJ, Adams GP, Creelman C, Singh J (2009) Effect of short- versus long proestrus on fertility in beef cattle after fixed-time artificial insemination *Annual Convention of American College of Theriogenology and the Society for Theriogenology*, Albuquerque, New Mexico USA

Creelman C, Dadarwal D, Singh J (2008) Effect of short versus long proestrous period along with low or high progesterone concentrations during the ovulatory wave on beef cattle fertility after FTAI *WCVM Poster Day* University of Saskatchewan Saskatoon Canada August 2008.

Study 8: Superovulatory response in a bovine model of reproductive aging

Related to the overall objective of the project, additional experiments were performed to examine the effect of ageing of beef cows on ovarian response to superstimulation. Two experiments were done to test the hypotheses that aging in cattle is associated with a reduced number of follicles recruited into an ovarian follicular wave, and a reduction in the ovarian response to gonadotropin treatment. Older cows (13–16 years of age) and their daughters (3–6 years of age) were treated with FSH for ovarian superstimulation four times over two consecutive years (31 and 33 superstimulations in old and young cows, respectively, experiments and years combined). In Experiment 1, ovulation was induced using pLH. In Experiment 2, cumulus-oocyte complexes were collected by ultrasonographic-guided follicle aspirations before expected ovulations. The ovarian follicular and ovulatory responses were monitored daily by ultrasonography. Combined data from Experiment 1 and 2 showed that fewer 2–5 mm follicles ($P < 0.01$) were detected at the expected time of follicular wave emergence in older cows than in their daughters. After superstimulation, older cows had fewer follicles ≥6 mm ($P < 0.01$), and tended ($P = 0.1$) to have fewer ovulations than their daughters (32±4 versus 40±3, respectively). There was a positive correlation in the response of individual cows to successive superstimulatory treatments ($r > 0.8$; $P < 0.0001$) and the number of detected ovulations from one year to the next ($r = 0.6$; $P = 0.04$). In conclusion, aging was

associated with fewer 2–5 mm follicles at follicular wave emergence and a lower follicular and ovulatory response after superstimulatory treatment. The follicular and ovulatory response after superstimulation was repeatable within individuals, regardless of age.

This work was published in Animal Reproduction Science. Please see the appended article for detials.

Malhi PS, Adams GP, Mapletoft RJ, **Singh J** (2008) Superovulatory response in a bovine model of reproductive aging *Animal Reproduction Science* **109** 100-109.

Study 9: Progress in understanding ovarian follicular dynamics in cattle

Based on this ADF grant and two previous ADF grants, we have published a review article in Theriogenology to highlights of our recent research. An abstract of this review follows.

The study of follicular dynamics began in the mid-20th century, but progress has been particularly rapid in the last two decades through the use of tools that have enabled serial, non-invasive examination. A brief overview of early oogenesis and folliculogenesis is provided as a backdrop to the evolution of our understanding of follicular dynamics during the bovine estrous cycle. Studies to date support the concept that the pair of ovaries acts as a single unit and influences follicular development primarily via systemic endocrine routes involving ovarian and uterine products, the gonadotropins, and their receptors. Dominant and subordinate follicles pass through growing, static and regressing phases that have distinct morphologic and biochemical characteristics; these changes are the basis of efforts focused on diagnosing and manipulating follicular status. An update of research progress highlights recent findings on the repeatability (predictability) within individuals of follicle recruitment and wave pattern (two- versus three-wave cycles), the relationship between oocyte competence and follicular status, and the dynamics of small follicles. Recent studies documented that wave emergence and follicular dominance are apparent earlier than previously reported, and on the basis of periodic endogenous FSH surges and the presence of FSH receptors, the hypothesis that follicles become progressively entrained to waves from the earliest stages of development is introduced. Lastly, recent studies comparing old cows and their young daughters provide a new understanding of the effects of aging on gonadotropins and ovarian steroids, follicular dynamics, ovarian response to synchronization, superstimulation, and oocyte competence.

Please see the following article in the Appendix for details

Adams GP, Jaiswal R, Singh J, Malhi PS (2008) Progress in understanding ovarian follicular dynamics in cattle *Theriogenology* **60** 72-80.

(c) **Conclusions and Recommendations** - Highlight significant conclusions based on the discussion and analysis provided in the previous section with emphasis on the project

objectives specified above; also provide recommendations for the application and adoption of the project results and identify any further research, development and communication needs, if applicable.

The following are the salient findings of the project:

- Long-duration of progesterone exposure during growth of the dominant follicle in lactating beef cows and beef heifers resulted in a larger diameter of the ovulatory follicle, but pregnancy rate was not significantly different than in the short-progesterone exposure group.
- Oocyte competence in superstimulated cows did not improve by shortening the duration of progesterone exposure during follicular growth. FSH starvation substantially reduced the ability of superstimulated follicles to ovulate.
- Low circulating progesterone concentrations during the growing phase of the ovulatory follicle resulted in a larger dominant follicle and a larger CL that produced more progesterone, with no significant effect on pregnancy rate.
- Higher doses of GnRH were more efficacious in inducing LH release in heifers, and exogenous estradiol increased LH release following treatment with GnRH. High concentrations of progesterone decreased both plasma LH concentrations and ovulation rates in heifers following treatment with GnRH.
- The progesterone environment (LH pulse-frequency) during the early growing phase after wave emergence did not influence oocyte competence in superstimulated heifers.
- The use of prostaglandin with or without exogenous progesterone treatment increased the percentage of heifers close to spontaneous puberty that ovulated following treatment.
- Shortening the period of proestrus resulted in dramatic decrease in pregnancy rate following fixed-time AI in beef cattle. A low progesterone environment during growth of the ovulatory follicle increased ovulatory follicle size and subsequent CL size and function, but it did not compensate for the effect of shortened proestrus on pregnancy rates.

Results of these studies were expected to increase fertility to AI in both beef and dairy cattle and help researchers to design effective, simplified and less expensive protocols for fixed-time artificial insemination. We thank the Saskatchewan Agricultural Development Fund for financially supporting the operational costs of this project. It is noteworthy that we were also supported by a cash contribution of $15,000 from Westgen Inc. and the veterinary pharmaceutical industry contributed the hormones needed for this project (in-kind contribution of more than $50,000).

(f) **Acknowledgements** - Include actions taken to acknowledge the Department's support for the project.

The funding support provided by ADF, Westgen Inc. and pharmaceutical industry has been acknowledged in the poster presentations at the Conferences and in the publications / manuscripts submitted.

(g) Literature Cited

Adams GP. 1999. Comparative patterns of follicle development and selection in ruminants. J Reprod Fertil Suppl 54:17-32.

Adams, G.P., 1994. Control of ovarian follicular wave dynamics in cattle: Implication for synchronization and superstimulation. Theriogenology 41, 25-30.

Adams, G.P., 1998. Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization & superstimulation. In: Proceedings of the Twentieth Congress of the World Association for Buiatrics, Sydney, Australia, vol. 2, pp. 595-605.

Adams, G.P., 1999. Comparative patterns of follicle development and selection in ruminants. J. Reprod. Fertil. Suppl. 54, Reproduction in Domestic Ruminants IV, 17-32.

Adams, G.P., 2000. Developments in the use of ultrasonography in buiatrics. In: Proceedings of the Congresso Nazionale della Societa Italiana di Buiatria, Stresa, Italy, 5-7 May, vol. XXXII, pp. 435-450.

Adams, G.P., Jaiswal, R., Singh, J., Malhi, P., 2008. Progress in understanding ovarian follicular dynamics in cattle. Theriogenology 69, 72-80.

Adams GP, Kot K, Ginther OJ. 1993. Selection of a dominant follicle and suppression of follicular growth in heifers. Animal reproduction science 30:259-271.

Adams GP, Matteri RL, Ginther OJ. 1992a. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. Journal of reproduction and fertility 96:627-640.

Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. 1992b. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. Journal of reproduction and fertility 94:177-188.

Beg MA, Bergfelt DR, Kot K, Wiltbank MC, Ginther OJ. 2001. Follicular-fluid factors and granulosa-cell gene expression associated with follicle deviation in cattle. Biol Reprod 64:432-441.

Bergfelt DR, Bo GA, Mapletoft RJ, Adams GP. 1997. Superovulatory response following ablation-induced follicular wave emergence at random stages of the oestrous cycle in cattle. Anim Reprod Sci 49:1-12.

Bergfelt DR, Lightfoot KC, Adams GP. 1994. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 42:895-907.

Bo GA, Adams GP, Nasser LF, Pierson RA, Mapletoft RJ. 1993. Effect of estradiol valerate on ovarian follicles, emergence of follicular waves and circulating gonadotropins in heifers. Theriogenology 40:225-237.

Bo GA, Adams GP, Pierson RA, Mapletoft RJ. 1995. Exogenous control of follicular wave emergence in cattle. Theriogenology 43:31-40.

Bo GA, Baruselli PS, Moreno D, Cutaia L, Caccia M, Tribulo R, Tribulo H, Mapletoft RJ. 2002. The control of follicular wave development for self-appointed embryo transfer programs in cattle. Theriogenology 57:53-72.

Fortune, J.E., 1993. Follicular dynamics during the bovine estrous cycle: A limiting factor in improvement of fertility? Anim. Reprod. Sci. 33, 111-125.

Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX, Kot K. 2001a. Follicle selection in

monovular species. Biol Reprod 65:638-647.
Ginther OJ, Bergfelt DR, Beg MA, Kot K. 2001b. Follicle selection in cattle: role of luteinizing hormone. Biol Reprod 64:197-205.
Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 2000a. Selection of the dominant follicle in cattle: role of estradiol. Biol Reprod 63:383-389.
Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. 2000b. Selection of the dominant follicle in cattle: role of two-way functional coupling between follicle-stimulating hormone and the follicles. Biol Reprod 62:920-927.
Ginther OJ, Kastelic JP, Knopf L. 1989a. Intraovarian relationship among dominant and subordinate follicles and corpus luteum in heifers. Theriogenology 32:787-795.
Ginther OJ, Knopf L, Kastelic JP. 1989b. Ovarian follicular dynamics in heifers during early pregnancy. Biology of reproduction 41:247-254.
Ginther OJ, Knopf L, Kastelic JP. 1989c. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. Journal of reproduction and fertility 87:223-230.
Ginther, O.J., Wiltbank, M.C., Fricke, P.M., Gibbons, J., Kot, K., 1996. Selection of the dominant follicle in cattle. Biol. Reprod. 55, 1187-1194.
Knopf L, Kastelic JP, Schallenberger E, Ginther OJ. 1989. Ovarian follicular dynamics in heifers: test of two-wave hypothesis by ultrasonically monitoring individual follicles. Domestic animal endocrinology 6:111-119.
Mapletoft RJ, Martinez MF, Adams GP, Kastelic JP, Burnley C. 1999. The effect of estradiol preparation on follicular wave emergence and superovulatory response in norgestomet-implanted cattle. Theriogenology 51:411.
Mapletoft RJ, Steward KB, Adams GP. 2002. Recent advances in the superovulation in cattle. Reprod Nutr Dev 42:601-611.
Pierson RA, Ginther OJ. 1987. Follicular populations during the estrous cycle in heifers I: influence of day. Animal reproduction science 124:165-176.
Pierson RA, Ginther OJ. 1988. Follicular populations during the estrous cycle in heifers III: time of selection of ovulatory follicle. Animal reproduction science 16:81-95.
Rawlings NC, Jeffcoate IA, Reiger DL. 1984. The influence of estradiol-17 and progesterone on peripheral serum concentrations of luteinizing hormone and follicles stimulating hormone on the ovariectomized ewe. Theriogenology 22:473-488.
Savio JD, Keenan L, Boland MP, Roche JF. 1988. Pattern of growth of dominant follicles during the oestrous cycle of heifers. Journal of reproduction and fertility 83:663-671.
Singh J, Dominguez M, Jaiswal R, Adams GP. 2004. A simple ultrasound test to predict the superstimulatory response in cattle. Theriogenology 62:227-243.

(h) **Appendices** - If necessary, include any materials supporting the previous sections, e.g. detailed data tables, maps, graphs, specifications.

Tables and graphs are included in the report.

(i) **Other** - (e.g. extension meetings; papers produced; conference presentations made; personnel involved; equipment bought; photos; project materials developed; expense statement).

Please see attached published articles, abstracts in conference proceedings and manuscripts submitted for publications

Published Papers

Pfeifer LF, Mapletoft RJ, Kastelic JP, Small JA, Adams GP, Dionello NJ, Singh J. (2009) Effects of low versus physiologic plasma progesterone concentrations on ovarian follicular development and fertility in beef cattle *Theriogenology* 72 1237-50.

Pfeifer LF, Siqueira LG, Mapletoft RJ, Kastelic JP, Adams GP, Colazo MG, Singh J (2009) Effects of exogenous progesterone and cloprostenol on ovarian follicular development and first ovulation in prepubertal heifers *Theriogenology* 72 1054-1064.

Malhi PS, Adams GP, Mapletoft RJ, Singh J (2008) Superovulatory response in a bovine model of reproductive aging *Animal Reproduction Science* 109 100-109.

Adams GP, Jaiswal R, Singh J, Malhi PS (2008) Progress in understanding ovarian follicular dynamics in cattle *Theriogenology* 60 72-80.

Abstracts

Dadarwal D, Mapletoft RJ, Adams GP, Creelman C, Singh J (2009) Effect of short- versus long proestrus on fertility in beef cattle after fixed-time artificial insemination *Annual Convention of American College of Theriogenology and the Society for Theriogenology*, Albuquerque, New Mexico USA

Dadarwal D, Mapletoft RJ, Adams GP, Creelman C, Singh J (2009) The effects of progesterone concentration and duration of proestrus on fertility in beef cattle after fixed-time artificial insemination *Proceedings of the Reproductive Science and Medicine Research Symposium* February 27, 2009 (page 7, oral presentation) and *Life Sciences Day* University of Saskatchewan Saskatoon Canada

Pfeifer LF, Siqueira LG, Mapletoft RJ, Adams GP, Singh J (2008) Effect of exogenous progesterone on follicular development and ovulation rate in prepubertal heifers *Proceedings of Annual Conference of SBTE (Brazilian Embryo Transfer Society)* Guarujá Brazil. August 21-24, 2008. Acta Scientiae Veterinariae 30: 457 (poster).

Creelman C, Dadarwal D, Singh J (2008) Effect of short versus long proestrous period along with low or high progesterone concentrations during the ovulatory wave on

beef cattle fertility after FTAI *WCVM Poster Day* University of Saskatchewan Saskatoon Canada August 2008.

Dias FCF, Costa E, Adams GP, Mapletoft RJ, Dochi O, Singh J (2008) Effect of length of progesterone exposure during the growing phase of the ovulatory follicular wave on oocyte competence after superovulatory treatment *Proceedings of the 16th International Congress on Animal Reproduction* Budapest Hungary, July 2008. *Reproduction in Domestic Animals* 43 37 (Abstract # P029)

Pfeifer LF, Mapletoft R, Adams G, Kastelic J, Small J, Dias F, Singh J (2008) The effect of progesterone on follicular development and pregnancy rate in beef cattle *Reproductive Science and Medicine Program Symposium* University of Saskatchewan Saskatoon Canada February 29, 2008 (oral presentation).

Pfeifer LF, Mapletoft R, Adams G, Kastelic J, Small J, Dias F, Singh J (2008) The effect of progesterone on follicular development and pregnancy rate in beef cattle *Proceedings of the 34th annual conference of the International Embryo Transfer Society* Denver Colorado USA January 5-9, 2008 Abstract # 2345. *Reproduction Fertility and Development* 20 p90 (poster)

Dias FCF, Stover BC, Mapletoft RJ, Kastelic J, Adams GP, Colazo M, Dochi O, Singh J (2007) Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate *Proceedings of the 53rd Annual Meeting of the Canadian Fertility and Andrology Society* Halifax NS Canada September 26-29, 2007 Abstract#TP16 (poster).

Chaîné K, Pfeifer LFM, Mapletoft RJ, Kastelic J, Colazo M and Singh J (2007) Ovulation induction in peri-pubertal heifers: Follicular dynamics and endocrine changes *The 2007 Merck-Merial Symposium* National Institutes of Health Bethesda USA August 02-04, 2007 and *WCVM Poster Day* University of Saskatchewan Saskatoon Canada September 6, 2007 (poster).

Dias FCF, Stover BC, Mapletoft RJ, Kastelic J, Adams GP, Colazo M, Dochi O and Singh J (2007) Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate in cattle *Reproductive Science and Medicine Research Symposium* (March 9, 2007) and *14th Life sciences and Health Sciences Research Conference* Saskatoon Saskatchewan Canada March 16, 2007 (oral presentation and poster)

Stover B, Dias FC, Mapletoft RJ, Kastelic J, Adams GP and Singh J (2006) Effect of length of progesterone exposure during ovulatory wave development on pregnancy rate *WCVM Poster Day* Saskatoon Saskatchewan Canada September 06-08, 2006 (poster).

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP (2006) Oocyte competence under different progestational environments *Proceedings of the 39th Annual Meeting of the Society for the Study of Reproduction* Omaha Nebraska USA July 29-August 1, 2006. Biology of Reproduction 2006 Special Issue p109, Abstr. # 166.

Manuscripts submitted for publication

Dias FCF, Stover BC, Mapletoft RJ, Kastelic J, Adams GP, Colazo M, Dochi O, Singh J (2010) Duration of progesterone exposure during development of the ovulatory follicle does not affect pregnancy rate in cattle

Dias FCF, Costa E, Adams GP, Mapletoft RJ, Dochi O, Singh J (2010) Effect of length of progesterone exposure during the growing phase of the ovulatory follicular wave on oocyte competence after superovulatory treatment

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP (2009) Oocyte competence under different progestational environments

Dias FCF, Colazo MG, Kastelic JP, Mapletoft RJ, Adams GP, Singh J (2009) Progesterone concentration, estradiol pre-treatment, and dose of GnRH affect GnRH-mediated LH release in beef heifers.

Final Report – ADF Project # 20050731

OPTIMIZING FERTILITY AND OOCYTE QUALITY AFTER ARTIFICIAL INSEMINATION IN BEEF CATTLE

Published Articles

with Saskatchewan ADF Funding Support

Available online at www.sciencedirect.com

ScienceDirect

Theriogenology

Theriogenology 72 (2009) 1237–1250

www.theriojournal.com

Effects of low versus physiologic plasma progesterone concentrations on ovarian follicular development and fertility in beef cattle

L.F.M. Pfeifer [a,b], R.J. Mapletoft [a], J.P. Kastelic [c], J.A. Small [d], G.P. Adams [a], N.J. Dionello [b], J. Singh [a,*]

[a] *University of Saskatchewan, Saskatoon, SK, Canada*
[b] *Universidade Federal de Pelotas, Department of Animal Science, Pelotas, RS, Brazil*
[c] *Agriculture and Agri-Food Canada Research Centre, Lethbridge, AB, Canada*
[d] *Agriculture and Agri-Food Canada Research Centre, Brandon, MB, Canada*

Received 9 December 2008; received in revised form 15 July 2009; accepted 29 July 2009

Abstract

The objective of this study was to determine the effects of low versus physiologic plasma progesterone concentrations during the ovulatory wave on fertility in cattle. Suckled beef cows (*Bos taurus*; n = 129) and pubertal heifers (*Bos taurus*; n = 150) at random stages of the estrous cycle were given a luteolytic dose of prostaglandin $F_{2\alpha}$ (500 μg cloprostenol; PGF) twice, 11 d apart. Ten days after the second PGF treatment, cattle were given estradiol benzoate im (1.5 and 1.0 mg for cows and heifers, respectively) and a progesterone-releasing intravaginal device (Cue-Mate) with a single pod containing 0.78 g progesterone (Day 0). Cattle in the low-progesterone group (n = 148) received a luteolytic dose of PGF on Day 0, whereas those in the high-progesterone (i.e., physiologic plasma concentrations) group (n = 131) were allowed to retain their corpora lutea. On Day 8, the Cue-Mate was removed, and PGF was given to both groups. Fifty-four hours to 56 h later, cattle received 12.5 mg of porcine LH (pLH) im and were concurrently artificially inseminated. The dominant follicle in the low-progesterone group was larger ($P < 0.001$) than that in the high-progesterone group on the day of insemination (14.9 ± 0.3 mm vs. 12.7 ± 0.3 mm, mean ± SEM). At 7 d after ovulation, the low-progesterone group had a larger corpus luteum (24.5 ± 0.54 mm vs. 21.9 ± 0.64 mm, $P < 0.01$) and higher plasma progesterone concentration (4.0 ± 0.3 vs. 3.1 ± 0.2, $P < 0.01$) than that of the high-progesterone group. However, pregnancy rates did not differ (79 of 148, 53.4%, and 70 of 131, 53.4%) for low- and high-progesterone groups, respectively). In summary, low circulating progesterone concentrations during the growing phase of the ovulatory follicle resulted in a larger dominant follicle and a larger CL that produced more progesterone, with no significant effect on pregnancy rate.
© 2009 Elsevier Inc. All rights reserved.

Keywords: Cattle; Corpus luteum; Ovarian follicular development; Progesterone; Synchronization of ovulation

1. Introduction

Control and manipulation of the estrous cycle in cattle is an important management tool to improve reproductive performance [1]. Despite some variation, protocols involving progestin-releasing devices have been used very successfully for estrous synchronization

* Corresponding author. Tel.: +1 306 966 7410; fax: +1 306 966 7405.
E-mail address: jaswant.singh@usask.ca (J. Singh).

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2009.07.019

and fixed-time artificial insemination (AI) in beef cattle. The efficiency of progestin devices facilitates breeding lactating beef cows [2], including those with unfavorable environmental conditions (e.g., high rates of postpartum anestrus) [3–6].

With improvements in the ability to control ovarian follicular development, the duration of progestin treatment in synchronization protocols has progressively decreased. The first studies using progesterone to synchronize the estrous cycle of cattle in the early 1940 s involved repeated administrations of progesterone for 14 d [7]. However, prolonged progesterone treatment reduces fertility at the subsequent estrus, apparently due to the development of persistent follicles and reduced oocyte competence [8,9]. In subsequent studies, giving estradiol in association with progestin treatment synchronized follicular development, enabling short duration (approximately 1 wk) progestin treatment and acceptable fertility to fixed-time AI [10].

Despite promising results with fixed-time AI protocols, ovarian follicular growth patterns and oocyte development have been shown to be influenced by variable gonadotropin release during progestin treatment [11–13]. Sanchez et al. [14] reported that an implant containing 3 mg norgestomet in mature heifers without a corpus luteum (CL) resulted in a luteinizing hormone (LH) pulse frequency typical of the follicular phase (approximately 1 pulse/h). However, two or four norgestomet implants suppressed LH pulse frequency to that of the luteal phase [15], supporting the contention that circulating progesterone concentrations modulate LH concentrations and pulsatility and, in turn, growth of the ovulatory follicle [16].

Circulating progesterone concentrations are known to affect follicular development [16] and oocyte quality [17–19], and several studies have shown that variations in circulating progesterone concentrations affect fertility after a synchronized breeding [12,13]. Low progesterone concentrations, and consequently high LH pulse-frequency [20], during the ovulatory wave were associated with decreased fertility attributed to premature oocyte maturation [21,22]. However, low progesterone concentrations promoted overgrowth of the dominant follicle [16], which may improve CL function [23] and fertility, as long as ovulation of a competent oocyte was achieved.

The objective of the current experiment was to elucidate the effects of plasma progesterone concentrations on follicular wave dynamics, hormone profiles, and fertility in suckled beef cows and pubertal heifers, wherein the life span of the dominant ovarian follicle was controlled by induction of ovulation. We tested the hypothesis that a low plasma progesterone concentration during selection and growth of the dominant follicle will increase the diameter of preovulatory dominant follicles, increase subsequent CL diameter and function, and improve pregnancy rate compared with that of physiologic plasma progesterone concentrations during the luteal phase.

2. Materials and methods

2.1. Animal treatments and ultrasonographic examinations

Suckled beef cows (*Bos taurus*; Hereford and Hereford × Charolais; n = 129) and beef heifers (*Bos taurus*; n = 150) were used. The cows were 3 to 14 yr of age, 60 to 75 d postpartum, 450 to 650 kg body weight, and were maintained at the University of Saskatchewan Goodale Research Farm, near Saskatoon, SK, Canada. The heifers were 14 to 16 mo of age, 310 to 430 kg body weight, and were maintained at two locations: Hereford and Hereford × Charolais heifers (n = 48) were maintained at the Goodale Research Farm (Location 1), and Angus heifers (n = 102) were maintained at the Agriculture and Agri-Food Canada Research Centre, Brandon, MB, Canada (Location 2). The animals were maintained on pasture, with ad libitum access to water, salt, and a mineral mixture.

Before the start of the experiment, ovarian function was assessed twice (10 d apart) by transrectal ultrasonography (Aloka SSD-900 with 7.5-MHz linear-array transducer; Aloka, Tokyo, Japan); only those cows and heifers in which a CL was detected at one or both examinations were included. The experimental design and treatment schedule are shown in Fig. 1. All cattle were given 500 μg cloprostenol im (PGF; Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) twice, 11 d apart, and allocated by age, location, parity, days postpartum (DPP, cows only), body condition score (BCS, 1 = emaciated and 9 = obese [24]), and body weight into two groups: high progesterone group (i.e., physiologic plasma concentrations; HP, n = 131; 70 heifers and 61 cows) and low progesterone group (LP, n = 148; 80 heifers and 68 cows). Ten days after the second PGF (Day 0), corresponding with approximately 5 to 8 d after ovulation, all cattle received an intravaginal progesterone-releasing device (Cue-Mate; Bioniche Animal Health, Belleville, ON, Canada). Concurrently, cows were given 1.5 mg im estradiol benzoate (Sigma Chemical Co., St. Louis, MO, USA) dissolved in canola oil, whereas heifers were given 1.0 mg im

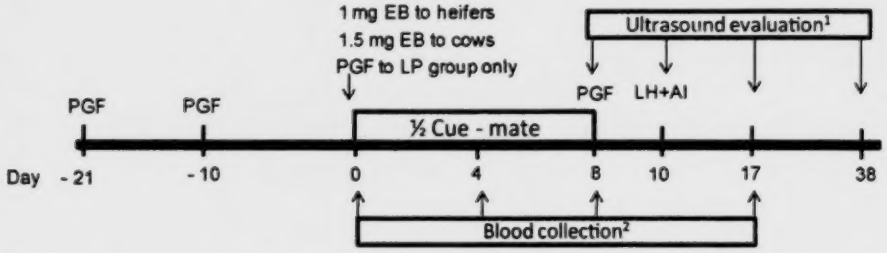


Fig. 1. Timeline for treatment of high progesterone (HP) and low progesterone (LP) groups. Cattle were given 500 μg cloprostenol (PGF) 11 d apart and assigned randomly into two groups 10 d after the second PGF (Day 0). On Day 0, cattle received a Cue-Mate with a single progesterone-releasing pod and a blank pod, and either 1.5 mg (cows; n = 129) or 1.0 mg (heifers; n = 150) estradiol benzoate (EB). In addition, cattle in the LP group were given PGF. On Day 8, the Cue-Mate was removed, and PGF was given to all cattle. At 54 to 56 h after Cue-Mate removal, cattle were given 12.5 mg porcine LH (Lutropin-V) and artificially inseminated (AI). [1]The dominant follicle diameter was measured ultrasonographically on Days 8 and 10, CL on Day 17, and pregnancy diagnosis was done on Day 38. [2]Plasma concentrations of progesterone were measured on Days 4, 8, and 17, and plasma concentrations of LH were measured on Days 0, 4, and 8.

estradiol benzoate. Each Cue-Mate was equipped with 1 progesterone-releasing pod (containing 0.78 g progesterone) and a second blank pod. It was expected that plasma progesterone concentrations <1 ng/mL would be achieved 2 d after insertion of these devices in cattle without a functional CL [25]. In addition, cattle in the LP group were given 500 μg cloprostenol im on Day 0 to achieve low plasma progesterone concentrations. In the HP group, cattle were allowed to retain their corpora lutea (i.e., no PGF treatment on Day 0) to maintain luteal-phase plasma progesterone concentrations. On Day 8, Cue-Mate devices were removed, and all cattle were given 500 μg cloprostenol im. Fixed-time artificial insemination was done 54 to 56 h later, and cattle were concurrently given 12.5 mg of porcine LH (pLH) im (Lutropin-V, Bioniche Animal Health). Estrus detection was performed by visual observation twice daily at 0700 and 1900; cattle detected in standing estrus between 0 and 36 h after Cue-Mate removal were inseminated 12 h after first detection of estrus and concurrently given 12.5 mg pLH.

Transrectal ultrasonography was done on Day 8 (Cue-Mate removal) and Day 10 (just before insemination) to determine the diameter of the dominant follicle, on Day 17 to determine the diameter of the CL and the ovulation rate, and on Day 38 to detect pregnancy. The diameter of the CL on Day 17 in heifers was measured only at Location 1.

2.2. *Blood sampling*

Blood samples were collected (caudal venipuncture) into heparinized 10-mL tubes (Vacutainer Systems; Becton-Dickinson, Franklin Lakes, NJ, USA) that were immediately centrifuged (1500 × *g* for 15 min); plasma was harvested and stored at −20 °C. Samples collected on Days 0, 4, and 8 were used to determine plasma LH concentrations, whereas those collected on Days 4, 8, and 17 were used to determine plasma progesterone concentrations.

2.3. *Daily ultrasonographic evaluations and intensive blood sampling*

On Day 0, a subset of six cows and six heifers per group was selected randomly to monitor ovarian follicular development and to measure plasma concentrations of gonadotropins and steroids. All ultrasonographic examinations were done by one operator, once daily from Day 0 to the day of ovulation, or to Day 12 (in the absence of ovulation). At each examination, a sketch of each ovary was made, and the diameter and location of follicles ≥3 mm in diameter were recorded [26]. Ovulation was defined as the disappearance (from one examination to the next) of a previously identified follicle ≥8 mm in diameter [27]. The day of wave emergence was defined retrospectively as the day when the dominant follicle was first detected at a diameter of 4 to 5 mm [26]. Blood samples were collected, as described, once daily from Day 0 to ovulation (or to Day 12) to measure plasma concentrations of LH, progesterone, estradiol, and follicle-stimulating hormone (FSH). In addition, blood samples were collected every 15 min for 8 h on Day 4 (i.e., expected day of wave emergence) to assess the pattern of LH release. Immediately before the frequent sampling period, each animal was fitted with an indwelling jugular catheter (Angiocath, 16 gauge, 8.26 cm; Becton-Dickinson Vascular Access, Sandy, UT, USA), held in place with Kamar adhesive glue (Kamar Products, Inc., Steamboat Springs, CO, USA),

Vetwrap Bandaging Tape (3 M, Animal Care Products, St. Paul, MN, USA), and 5-cm elastic medical tape. The catheter was filled with heparinized saline (0.9% sodium chloride with 0.1% heparin), and the end of the catheter was sealed with a cap. Before sample collection, the heparinized saline solution was removed and discarded, and after collection, catheters were flushed with fresh heparinized saline to prevent blood clot formation.

2.4. Hormone assays

Plasma LH concentrations were determined by double-antibody radioimmunoassay [28] and expressed in terms of National Institute of Diabetes and Digestive and Kidney Disease rabbit bovine-LH antiserum (NIDDK-bLH-4). The minimum detection limit was 0.06 ng/mL, with a standard curve ranging from 0.06 to 8.0 ng/mL. Intra-assay coefficients of variation were 8.7% and 7.1% for low (0.90 ng/mL) and high (2.03 ng/mL) LH reference samples, whereas interassay coefficients of variation were 8.5% and 9.2% for low and high LH, respectively.

Plasma progesterone concentrations were evaluated with a solid-phase radioimmunoassay (Coat-a-Count; Diagnostic Products Corporation, Los Angeles, CA, USA), with a minimum detection limit of 0.1 ng/mL. Progesterone was extracted with 3 mL hexane from 200 μL aliquots of plasma. The intra-assay coefficients of variation were 12.3% and 8.2%, whereas the interassay coefficients of variation were 15.0% and 14.0% for low (0.24 ng/mL) and high (0.93 ng/mL) progesterone concentrations, respectively.

Plasma concentrations of estradiol were measured in duplicate (single assay) with a modified human double-antibody RIA Kit (Coat-a-Count; Diagnostic Products Corporation) [29], using the procedure described by Singh et al. [30]. The minimum detection limit was 0.5 pg/mL, and the intra-assay coefficient of variation was 10.8% (23.3 pg/mL). A reference sample (23.3 pg/mL) was included (10 times, distributed throughout the assay) and used to determine the intra-assay CV (10.8%).

Plasma FSH concentrations were determined with a liquid-phase antibody radioimmunoassay [31]. The first antibody used was NIDDK rabbit ovine-FSH antiserum (anti-oFSH-1), and FSH concentrations were expressed in terms of United States Department of Agriculture rabbit bovine-FSH antiserum (USDA-bFSH-1). The range of the standard curve was 0.13 to 16.0 ng/mL, the minimum detection limit was 0.13 ng/mL, and the intra-assay coefficients of variation were 4.7% and 4.5% for low (2.7 ng/mL) and high (5.9 ng/mL) FSH concentrations, respectively.

2.5. Statistical analyses

All statistical analyses were performed with SAS 9.0 (SAS Institute Inc., Cary, NC, USA). In the initial analyses of single-point measurements (i.e., maximal diameter of the ovulatory follicle on Days 8 and 10, interval to ovulation, growth rate of the ovulatory follicle, and diameter of the dominant follicle at selection), the statistical models included category (heifer vs. cow), treatment (LP vs. HP) and their interactions, and location, which was considered a random effect in the model. Within the cows category, the effects of age, parity, days postpartum (DPP), body condition score, and body weight had no significant effects in the model; therefore, they were excluded from the final statistical model. For these single-point measurements, two-way ANOVA was used to detect the effect of category (heifer vs. cow), treatment (LP vs. HP), and their interactions. Furthermore, Pearson's correlations were determined for the following pairs of end points: CL diameter and progesterone concentrations on Day 17; dominant follicle diameter on Day 10 and progesterone concentrations on Day 17; and plasma concentrations of LH and estradiol on Days 4 to 8. Logistic regression analyses were used to examine the effects of treatment (LP and HP) and category (heifers and cows) on rates of ovulation and pregnancy.

For plasma concentrations of progesterone on Days 4, 8, and 17 and of LH on Days 0, 4, and 8, repeated measures ANOVA, using the MIXED procedure, was used to evaluate the main effects of treatment, category, and time (day of sampling) and their interactions [32]. Because there were three-way interactions (progesterone, $P = 0.0056$; LH, $P = 0.0037$), four separate analyses (for each hormone and each category) were done. When significant interactions were detected, the least significant difference was used to locate differences. It is noteworthy that the ANOVA included an "estimate" statement to calculate contrasts between sample days for LP versus HP; in essence, this analysis compared, between days, the magnitude of the differences between the two treatments.

For the subset of cattle subjected to intensive measurements, mean plasma LH concentrations and frequency of LH pulses (pulses/8 h) were calculated from samples collected at 15-min intervals. An LH pulse was defined as an increase in LH concentration that exceeded the previous nadir by two intra-assay standard deviations [33]. Data from intensive blood collection to characterize the pattern of LH release (basal, mean, pulse frequency, and LH pulse amplitude) were also analyzed by two-way ANOVA to detect the effects of category, treatment, and

their interaction. For follicle diameter and hormonal profiles in cattle that were intensively examined, repeated measures ANOVA, using the MIXED procedure, was used to evaluate the main effects of treatment, category, and time (day) and their interactions, with a least significant difference used to locate differences. Repeated measures of plasma concentrations of progesterone, LH, FSH, and estradiol were analyzed over three periods: Days 0 to 4 (from start of progesterone treatment to follicular wave emergence), Days 4 to 8 (from follicular wave emergence to the end of progesterone treatment), and Days 8 to 13 (from the end of progesterone treatment to ovulation).

3. Results

3.1. Pregnancy rates and ovarian characteristics in all cattle

Reproductive performance and ovarian characteristics are summarized in Table 1. Overall, neither pregnancy rates nor ovulation rates differed between treatment groups (HP vs. LP) or between categories (cows vs. heifers). The proportion of cattle detected in estrus before scheduled fixed-time AI (mean ± SEM, 36.0 ± 7.6 h after Cue-Mate removal) was higher in the LP group (37 of 148, 25%) than that in the HP group (17 of 131, 13%; P = 0.014). When the data were analyzed separately for heifers and cows, the proportion of cows detected in estrus before fixed-time AI tended to be higher in the LP group (13 of 68, 19.1%) than that in the HP group (5 of 61, 8.2%; P = 0.08). Similarly, the proportion of heifers detected in estrus before fixed-time AI tended to be higher for the LP group (24 of 80, 30.0%) than that in the HP group (12 of 70, 17.1%; P = 0.08). There were no differences in pregnancy rates between cattle that were detected in estrus before scheduled fixed-time AI (51.6%) and those inseminated at a fixed-time (42.46%; P = 0.39), and there was no interaction of category and group (P = 0.26).

The diameter of the dominant follicle was larger in the LP group than that in the HP group at Cue-Mate removal (P = 0.002) and at insemination (P = 0.001). The diameter of the dominant follicle at insemination was also larger in cows than that in heifers (P = 0.001). The diameter of the CL on Day 17 was larger (P = 0.004) in the LP group than that in the HP group. There were significant positive correlations between the diameter of the dominant follicle at AI and the diameter of the CL on Day 17 (r = 0.52, P < 0.001) and between CL diameter and plasma progesterone concentration on Day 17 (r = 0.31, P = 0.002).

3.2. Hormone concentrations in all cattle

Plasma concentrations of progesterone and LH in heifers and cows are shown in Figs. 3 and 4. For both hormones in both categories of cattle, there was a significant treatment by time interaction. Consistent with the experimental design, plasma progesterone concentrations were significantly lower in LP cattle than that in HP cattle on Days 4 and 8. On Day 17, progesterone was significantly higher in LP heifers than that in HP heifers; in cows, these concentrations (3.4 ± 0.2 and 2.7 ± 0.2 ng/mL, respectively) had a similar trend, but the difference was not significant. For unknown reasons, plasma LH concentrations were significantly lower in HP versus LP heifers on Day 0, with no significant differences between the two treatments on Day 0 in cows. On Days 4 and 8, plasma LH concentrations were significantly higher in the LP group than that in the HP group in both heifers and cows.

Table 1
Reproductive end points in cows and heifers (category) with either low (LP) or high (HP) progesterone (treatment).

End point	Heifer		Cow		P value*		
	LP	HP	LP	HP	Trt	Cat	Trt by Cat
Pregnancy rate, %[‡]	55 (44/80)	50 (35/70)	51.5 (35/68)	57.4 (35/61)	0.9	0.81	0.37
Ovulation rate, %[‡]	83.75 (67/80)	77.14 (54/70)	81.4 (55/68)	84.0 (51/61)	0.64	0.76	0.33
Diameter of dominant follicle (DF) at Cue-Mate removal, mm	12.20 ± 0.34^{aA}	10.19 ± 0.55^{bB}	14.3 ± 0.51^{aC}	12.3 ± 0.34^{bA}	0.002	0.07	0.93
Diameter of dominant follicle (DF) at AI, mm	13.57 ± 0.35^{aA}	11.49 ± 0.48^{bB}	16.5 ± 0.52^{aC}	14.2 ± 0.34^{bA}	0.001	0.001	0.76
CL diameter 7 d after AI, mm	24.17 ± 0.83^{aA}	20.79 ± 1.06^{bB}	24.7 ± 0.69^{aA}	22.2 ± 0.76^{bAB}	0.004	0.3	0.64

*P values from statistical analyses for treatment (Trt; LP vs. HP), category (Cat; cows vs. heifers), and treatment by category interaction (Trt by Cat) are provided in last three columns.

[‡] Pregnancy rate and ovulation data were analyzed by logistic regression; the remaining data were analyzed by two-factor ANOVA.[a,b]Within a category, values without a common superscript differed between treatments.[A,B]Values without a common superscript differed between categories.

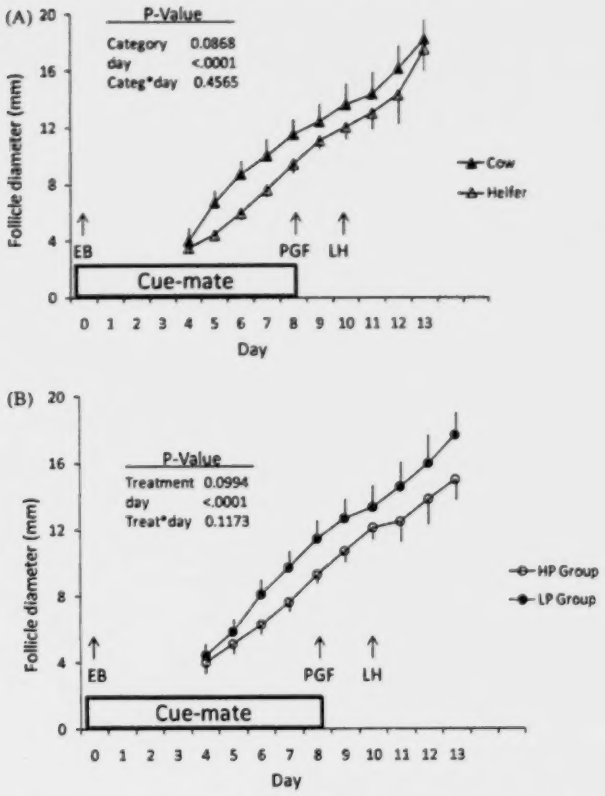


Fig. 2. Mean (±SEM) profiles of the dominant follicle from the subset of cattle that were examined daily by ultrasonography (n = 22): (A) cows versus heifers; (B) high progesterone (HP) versus low progesterone (LP) groups. For each group, data were centralized to the day of follicular wave emergence. Timing of estradiol benzoate (EB), progesterone implants (Cue-Mate), cloprostenol (PGF), and luteinizing hormone (LH) treatments are indicated by arrows.

Probability values for contrasts between sample days for LP versus HP are shown in Figs. 3 and 4. Most of the contrasts were significant, with the exception of Days 4 versus 8 for plasma progesterone concentrations in cows, and Days 4 versus 8 and 0 versus 4 for plasma LH concentrations in cows and heifers, respectively.

3.3. Daily ultrasonographic evaluations

One cow from each group lost the Cue-Mate during the treatment, and the cow was excluded from the analyses. Based on daily ultrasonographic examinations (n = 11/group), the interval from PGF treatment to ovulation was shorter ($P < 0.001$) in the LP group (60 ± 0.0 h) than that in the HP group (74.4 ± 3.9 h) and shorter in heifers (64.0 ± 2.6 h) than that in cows (72.0 ± 4.5 h). Furthermore, for this end point, there was a treatment-by-category interaction (P ± 0.02); cattle in the LP group ovulated earlier than did those in the HP group (heifers, 60 ± 0 h vs. 68 ± 5.1 h, P = 0.05; cows, 60 ± 00 h vs. 84 ± 0 h, P = 0.001).

There was no effect of treatment (P = 0.95), category (P = 0.57), or treatment-by-category interaction (P = 0.17) on the growth rate of the dominant follicle (combined growth rate = 1.8 ± 0.08 mm/d; n = 22). Similarly, there was no effect of treatment (P = 0.75), category (P = 0.54), or treatment-by-category interaction (P = 0.54) on the diameter of the dominant follicle at selection (combined for all cattle, size of dominant follicle at selection = 7.9 ± 0.12 mm; n = 22).

The dominant follicle development from emergence to ovulation tended to differ between categories (P = 0.09) and treatments (P = 0.1; Fig. 2A, B). The dominant follicle grew from 4.3 ± 0.3 mm on Day 4 (expected day of wave emergence) to 13 ± 0.8 mm (just before ovulation) in HP heifers, from 4.6 ± 0.6 to 15.6 ± 1.2 mm in LP heifers, from 6.0 ± 1.3 to 16.0 ± 2.0 mm in HP cows, and from 6.4 ± 1.3 to 16.8 ± 1.7 mm in LP cows.

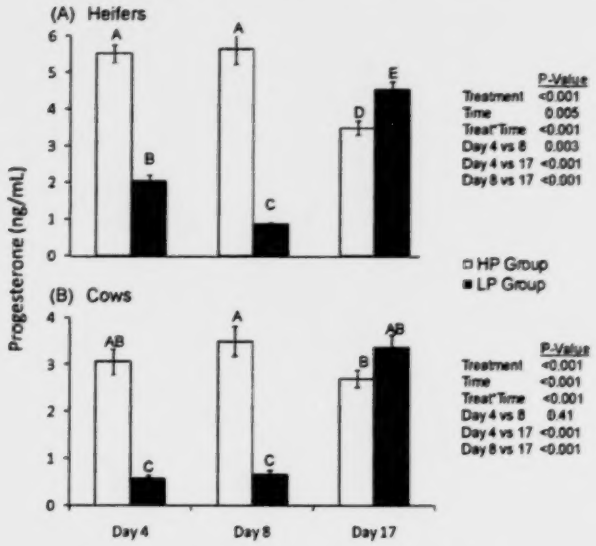


Fig. 3. Mean (±SEM) plasma progesterone concentrations in (A) beef heifers and (B) cows in high progesterone (HP) and low progesterone (LP) treatment groups on Day 4 (expected day of wave emergence; Day 0 = Day of estradiol benzoate injection + Cue-Mate insertion), Day 8 (Cue-Mate removal), and Day 17 (7 d after AI). Superscripts Show the difference among groups and days.

3.4. Intensive blood sampling

Progesterone profiles from daily blood samples are shown in Fig. 5 (n = 11 per group). There was an effect of treatment (P = 0.001) and a treatment-by-day interaction (P = 0.034) for progesterone concentrations from Day 4 (wave emergence) to Day 8 (Cue-Mate removal); mean progesterone concentrations during this interval were 1.1 ± 0.41 ng/mL and 3.8 ± 0.41 ng/mL for the LP and HP groups, respectively. In contrast, there was a category effect (P = 0.002) and a category-by-day interaction (P < 0.001) after Cue-Mate removal

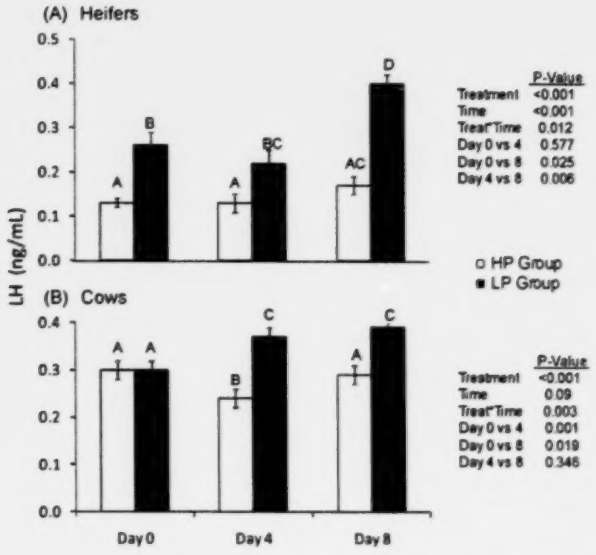


Fig. 4. Mean (±SEM) plasma LH concentrations in (A) heifers and (B) cows under high and low progesterone treatment on Day 0 (Cue-Mate insertion), Day 4 (expected day of follicular wave emergence), and Day 8 (Cue-Mate removal). Superscripts show the difference among groups and days.

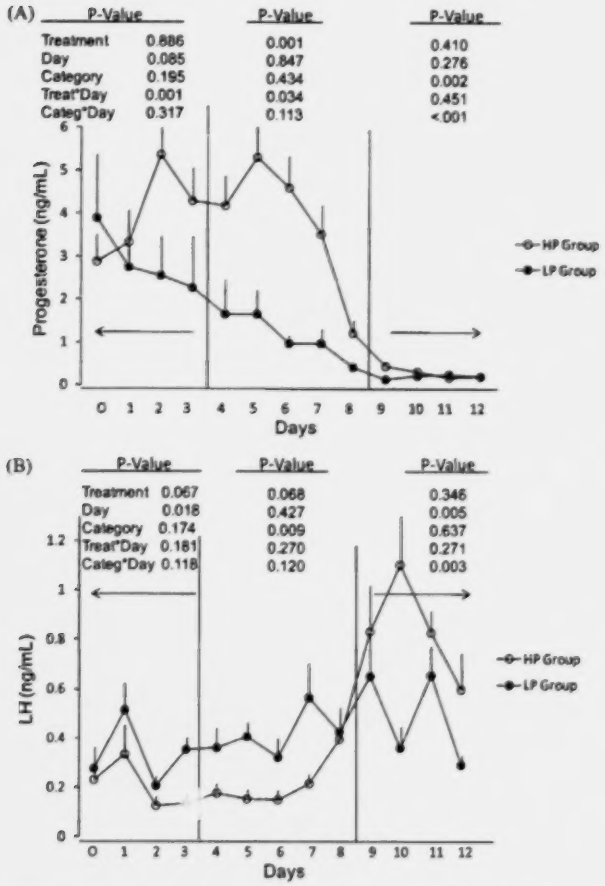


Fig. 5. Mean (±SEM) plasma concentrations of (A) progesterone (P4) and (B) LH in cattle in the high progesterone group (HP; ○) and low progesterone group (LP; ●). Data were analyzed for three time periods: before expected follicular wave emergence (Days 0 to 4); from the expected follicular wave emergence to Cue-Mate removal (Days 4 to 8); and from Cue-Mate removal to ovulation (Days 8 to 12).

(Fig. 5A); the mean progesterone concentrations were 0.6 ± 0.06 ng/mL and 0.3 ± 0.06 ng/mL for the LP and HP heifers and were 0.3 ± 0.07 ng/mL and 0.2 ± 0.07 ng/mL for LP and HP cows.

There was a tendency for a treatment effect (P = 0.068) and category effect (P = 0.009) for LH concentrations from wave emergence to Cue-Mate withdrawal (Fig. 5B); averaged over Days 4 to 8, LH

Table 2
Characteristics of plasma LH concentration (mean ± SEM) in cows and heifers given high (HP) or low (LP) progesterone treatments on Day 4 (expected time of wave emergence; Day 0 = Day of estradiol injection + Cue-Mate insertion). Blood samples were collected every 15 min for 8 h.

	Heifers		Cows		P value		
	LP	HP	LP	HP	Trt	Cat	Trt by Cat
Number of cattle	6	6	5	5			
Basal secretion, ng/mL	0.21 ± 0.02	0.13 ± 0.01	0.26 ± 0.04	0.21 ± 0.02	0.01	0.02	0.63
Mean, ng/mL	0.31 ± 0.07	0.18 ± 0.03	0.32 ± 0.03	0.23 ± 0.03	0.05	0.65	0.77
Pulse frequency, pulses/8 h	3.66 ± 0.8	2.5 ± 0.34	3.5 ± 0.86	2 ± 0.71	0.21	0.62	0.81
Pulse amplitude, ng/mL	1.28 ± 1.06	0.58 ± 0.36	0.23 ± 0.02	0.20 ± 0.08	0.64	0.43	0.61

*P values from statistical analyses for treatment (Trt; LP vs. HP), category (Cat; cows vs. heifers), and treatment by category interaction (Trt by Cat) are provided in last three columns.

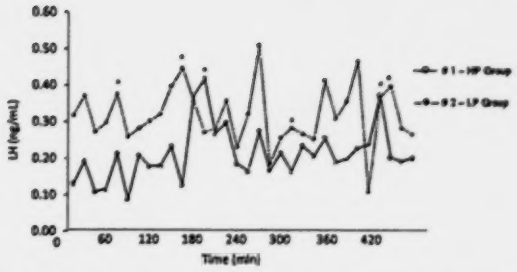


Fig. 6. Plasma concentrations of LH in two representative individuals from the high progesterone (Heifer # 1) and low progesterone (Heifer # 2) groups on Day 4 after Cue-Mate insertion. Blood was collected every 15 min for 8 h. Pulses of LH are indicated by asterisks. These representative animals for each treatment group were chosen based on the LH mean and LH pulse frequency that best represented the average of the group.

concentrations were 0.4 ± 0.04 ng/mL and 0.2 ± 0.04 ng/mL for the LP and HP groups, respectively. After Cue-Mate removal, there was an effect of day ($P = 0.005$) and a category-by-day interaction ($P = 0.003$). The LH secretion pattern assessed by 8-h intensive blood sampling on Day 4 is summarized in Table 2. The LP group had higher basal ($P = 0.01$) and mean LH ($P = 0.05$) concentrations than that of the HP group. No treatment effect or interaction was detected in LH pulse frequency or amplitude over the 8-h interval. Examples of LH secretory profiles for individual animals in each treatment group are shown in Fig. 6.

Daily measurements of estradiol and FSH were analyzed over three time intervals (Fig. 7). There was an effect of day on estradiol concentrations from Days 0 to 3 ($P < 0.01$), a category effect during all periods, and a treatment-by-day interaction from Days 4 to 8 ($P = 0.002$). Cows had higher estradiol concentrations than that of heifers during the first and the second intervals, however during the third interval (after Cue-Mate removal), heifers had higher estradiol concentrations than that of cows. There was a category-by-day interaction only during Days 0 to 3 (Fig. 7A). From Days 4 to 8, mean estradiol concentrations were 15.7 ± 1.4 pg/mL and 9.6 ± 1.4 pg/mL for the LP and HP groups, respectively. There was a positive correla-

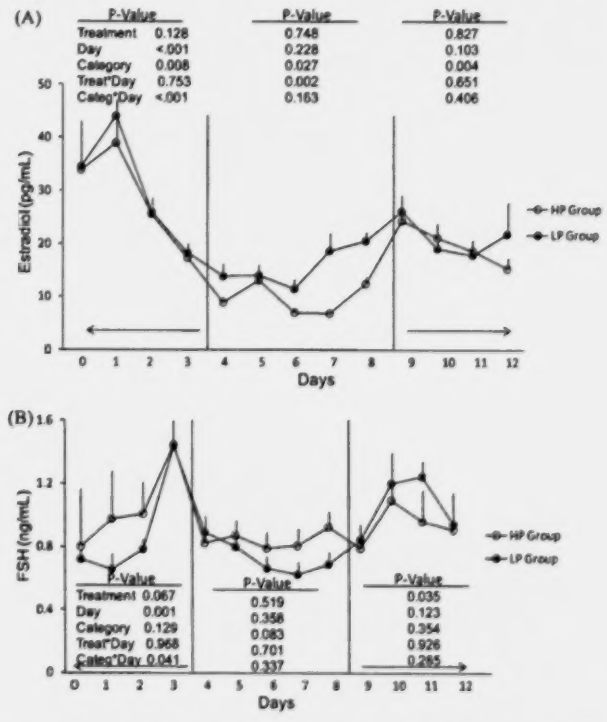


Fig. 7. Mean (±SEM) plasma concentrations of (A) estradiol (E2) and (B) FSH in cattle in the high progesterone group (HP; ○) and low progesterone group (LP; ●). The data were analyzed by repeated measures ANOVA for three time intervals: before expected follicular wave emergence (Days 0 to 4); from the expected follicular wave emergence to Cue-Mate removal (Days 4 to 8); and from Cue-Mate removal to ovulation (Days 8 to 12).

tion between LH and estradiol concentrations from Days 4 to 8 (during dominant follicular growth; $r = 0.73$, $P < 0.0001$).

During the first period of FSH evaluation (Days 0 to 3), there was a day effect ($P = 0.001$) and category-by-day interaction ($P = 0.04$). During the second period (Days 4 to 8), there were no significant effects or interactions, and during the last period (Days 8 to 12), there was only a significant effect of treatment on FSH secretion ($P = 0.03$).

4. Discussion

The results of the current study supported the hypothesis that reduced plasma progesterone concentrations during selection and growth of the dominant follicle will increase preovulatory follicle diameter and subsequent CL diameter and function; however, the hypothesis that this will improve pregnancy rate compared with that of physiologic plasma progesterone concentrations was not supported. Ovulation and pregnancy rates after insemination did not differ, regardless of whether plasma progesterone concentrations during growth of the ovulatory follicle were at reduced or physiologic concentrations. This was perplexing but consistent with other studies in which progestin treatments have been used to synchronize ovulation in postpartum cows, with pregnancy rates that ranged from 45% to 70% [34–39]. Clearly, in the current study with more than 275 cattle, a low plasma progesterone milieu during the growing and preovulatory phases of the dominant follicle did not have deleterious effects on oocyte competence in either lactating postpartum beef cows or beef heifers. It is also noteworthy that the life span of the ovulatory follicle under both low and physiologic progesterone concentrations was <8 d from wave emergence to ovulation, thereby preventing development of a persistent follicle and aged oocyte.

In the current study, ~20% of cattle were detected in estrus before the scheduled insemination time (54 to 56 h after Cue-Mate removal), especially in the LP group, which likely would have reduced pregnancy rates after fixed-time AI. However, by design, the study included early estrus detection followed by AI, thereby optimizing fertility and minimizing any confounding effects that might have resulted from strict adherence to the fixed-time AI schedule. Obviously, if reduced progesterone protocols were developed for future use, ovulation times would have to be determined before strict adherence to a fixed-time AI schedule could be recommended.

The LP group ovulated earlier than did the HP group; the interval from progestin removal to ovulation in the LP group seemed earlier than that reported in other studies [36,40,41]. The LP group had higher LH concentrations during dominant follicle growth, and presumably earlier induction of both LH receptors and ovulatory competence [42]. Nonetheless, Colazo et al. [37] detected no difference in the interval from CIDR removal to ovulation of heifers synchronized with various concentrations of progesterone.

The current study provided further evidence that synchronization protocols using physiologic or subphysiologic progestin concentrations can yield high pregnancy rates. In another study involving previously used CIDR inserts for fixed-time AI protocols in suckled beef cows, the numbers of times the CIDR had been used affected pregnancy rate [37]. Cows synchronized with a once-used CIDR had a higher pregnancy rate than that of cows synchronized with a twice-used CIDR (62.4% vs. 48.4%, respectively). However, in the same study [37], the authors reported no differences in the pregnancy rates of heifers receiving one new, one once-used, one twice-used, or two twice-used CIDR inserts. The authors speculated that some cows given a twice-used CIDR may have ovulated earlier than did cows given a CIDR containing more progesterone and consequently failed to become pregnant after fixed-time AI.

As the dominant follicle grew in different progesterone environments, LH secretion also differed between groups. As expected, the use of single progesterone-releasing pod on the Cue-Mate, with a blank pod on the second arm, markedly reduced plasma progesterone concentrations in the LP group. The high LH secretory activity in the LP group was apparently responsible for the greater growth of the dominant follicle. Because subluteal concentrations of progesterone have been associated with an increase in LH pulse frequency [20,43], the higher frequency of LH pulses apparently stimulated dominant follicle growth and estradiol secretion by the follicle [44]. Indeed, the LP group had higher LH secretory activity during progesterone treatment, and there was a positive correlation between LH and estradiol secretion from Days 4 to 8 of treatment. As LH stimulates steroidogenesis [20,45] in granulosa cells, the high estrogenic activity of the dominant follicle enhances the expression of the genes for aromatase, 3β-hydroxy-steroid dehydrogenase, and FSH receptors and the acquisition of LH receptors in granulosa cells [42,46–48]. In turn, the LP group had a larger dominant follicle at both Cue-Mate removal and at AI, and consequently a larger CL than that of the HP

group. In addition, there was a positive correlation between the diameter of the ovulatory follicle, CL, and progesterone concentrations 7 d after AI. Therefore, the structure and function of the CL were clearly associated with characteristics of the dominant follicle. A larger CL and greater progesterone concentration may increase fertility, as increased progesterone concentrations in the early luteal phase augment embryo development and establishment of pregnancy [49]. Treatment with equine chorionic gonadotropin (eCG) at the end of a progestin treatment has also been shown to result in a larger ovulatory follicle, a larger CL, higher progesterone concentrations after ovulation [50], and increased pregnancy rates after AI [51–53]. Despite the differences between groups in preovulatory follicle diameter, the ovulation rate did not differ (~80% for both groups). Therefore, we inferred that the pregnancy rate is more influenced by the ovulation rate than by the diameter of the ovulatory follicle.

The development of a normal CL capable of maintaining pregnancy depends on adequate numbers of granulosa cells, LH receptors on granulosa and theca cells, and granulosa cells capable of synthesizing adequate amounts of progesterone after luteinization [54]. Consequently, it would seem important to optimize ovulatory follicle size; small follicles have fewer granulosa cells prior to ovulation, resulting in fewer large luteal cells in the newly formed CL [23]. In the current study, it is likely that growth of the dominant follicle in the HP group was sufficient to mask any benefits of oocyte competence/luteal function due to a low progesterone milieu.

The difference in the diameter of the dominant follicle in LP and HP groups at the time of Cue-Mate removal was 2 mm. An apparently similar difference (1.6 mm) between heifers in low and high progesterone groups was reported by Carvalho et al. [12]. In a 7-d progestin synchronization protocol with estradiol cypionate to induce the new follicular wave and either estradiol or gonadotropin-releasing hormone (GnRH) to induce the ovulation, ovulatory follicles of 13.0 ± 0.5 mm and 14.1 ± 0.4 mm in diameter, respectively, were observed [40]. Obviously, it is not only the concentration of progesterone that affects the development of the ovulatory follicle but also the interval from follicular wave emergence to progestin withdrawal [55], the duration of progestin administration [55], the follicle age [56], and the treatment used to induce follicular wave emergence (i.e., esters of estradiol or GnRH) [40,56].

The diameter of the dominant follicle at selection was similar for both treatment groups and seemed similar to that reported for dairy heifers (8.3 ± 0.2 mm) but was apparently smaller than that reported for dairy cows (9.8 ± 0.3 mm) [57]. The physiologic growth rate of antral follicles during the rapid growth phase may be up to 2 mm/d [45], similar to that in the current study; however, others have reported growth rates of 1.2 ± 0.1 mm/d and 0.9 ± 0.1 mm/d for low- and high-progesterone groups in crossbred heifers, respectively [12], and 1.2 ± 0.08 mm/d for dairy cows [57].

The greater estradiol concentrations during the progesterone treatment period (Days 0 to 8) in cows than that in heifers highlighted the influence of progesterone on LH pulsatility and consequently on estradiol synthesis. However, in the absence of progesterone, heifers had higher estradiol concentrations than that in cows [57]. Although progesterone has been reported to be the primary feedback regulator of LH secretion in adult cattle [43], one study reported the failure of progestin treatment to suppress LH secretion [58] and another reported that progesterone enhanced the pulsatile release of LH in prepubertal heifers [15]. However, based on the current and previous studies [14,20,43], LH secretion depends on progesterone concentrations. Indeed, higher LH secretion and pulsatility occurred in the LP group than that in the HP group during progesterone treatments. The HP group had higher concentrations of progesterone during progesterone treatment, reflecting the presence of progesterone from the CL and the Cue-Mate, whereas the progesterone in the LP group came only from the Cue-Mate per se. The difference in LH secretion on Days 4 and 8 was attributed to circulating progesterone concentrations. That the LP group had higher LH concentrations on Day 0 could be related to the effectiveness of the second PGF to induce ovulation or to the timing of ovulation. We expected the CL to be 5 to 8 d old at Cue-Mate insertion; the difference in progesterone production by the CL could have affected the LH secretion by negative feedback. However, it is noteworthy that the treatments were effective in creating the endocrine environment by the time of expected wave emergence (Day 4) according to our experimental design (i.e., HP groups had lower plasma LH concentrations than that of LP groups).

In summary, although low circulating progesterone concentrations during follicular wave emergence, selection, and growth of the dominant follicle increased diameter of the ovulatory follicle, and consequently the diameter of the resulting CL and plasma progesterone concentrations, no effect on fertility was detected. We inferred that the mechanisms that regulated oocyte

quality and the establishment of pregnancy were not entirely dependent on circulating progesterone concentrations. However, it was noteworthy that in the absence of prolonged follicle dominance, the low progesterone environment did not decrease fertility.

Acknowledgments

The study was supported by research grants from the Saskatchewan Agriculture Development Fund, Natural Science and Engineering Council of Canada, and Westgen. Luiz Pfeifer was supported by the CAPES scholarship by the Education Ministry of Brazil. The authors thank Bioniche Animal Health for Cue-Mate and Lutropin and Schering-Plough Animal Health for Estrumate. The authors also thank Drs. Fernanda Caminha, Marcelo Martinez, and Luiz Siqueira for help with data collection and personnel from Agriculture and Agri-Food Canada, Research Centre (Brandon, MB, Canada) and from the Goodale Research Farm for providing cattle and technical assistance. Portions of these data were presented at the Annual Meeting of the International Embryo Transfer Society, January 2008.

References

[1] Kuroiwa T, Ishibashi A, Fukuda M, Kim S, Tanaka T, Kamomae H. Estrus synchronization and conception rate after a progesterone releasing intravaginal device (PRID) treatment from the early luteal phase in heifers. J Reprod Dev 2005;51:669–73.

[2] Tauck SA, Wilkinson JR, Olsen JR, Janitell JN, Berardinelli JG. Comparison of controlled internal drug release device and melengesterol acetate as progestin sources in an estrous synchronization protocol for beef heifers. Theriogenology 2007;68:162–7.

[3] Foote WD, Hauser ER, Casida L. Influence of progesterone treatment on post-partum reproductive activity in beef cattle. J Anim Sci 1958;17:1218.

[4] Patterson DJ, Corah LR, Kiracofe GH, Stevenson JS, Brethour JR. Conception rate in Bos taurus and Bos indicus crossbred heifers after postweaning energy manipulation and synchronization of estrus with melengestrol acetate and fenprostalene. J Anim Sci 1989;67:1138–47.

[5] Odde KG. A review of synchronization of estrus in postpartum cattle. J Anim Sci 1990;68:817–30.

[6] Larson LL, Ball PJ. Regulation of estrous cycles in dairy cattle: a review. Theriogenology 1992;38:255–67.

[7] Jochle W. Forty years of control of the oestrous cycle in ruminants: progress made, unresolved problems and the potential impact of sperm encapsulation technology. Reprod Fertil Dev 1993;5:587–94.

[8] Mihm M, Baguisi A, Boland MP, Roche JF. Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. J Reprod Fertil 1994;102:123–30.

[9] Anderson LH, Day ML. Acute progesterone administration regresses persistent dominant follicles and improves fertility of cattle in which estrus was synchronized with melengestrol acetate. J Anim Sci 1994;72:2955–61.

[10] Bo GA, Adams GP, Pierson RA, Mapletoft RJ. Exogenous control of follicular wave emergence in cattle. Theriogenology 1995;43:31–40.

[11] McDougall S, Compton CW, Anniss FM. Effect of exogenous progesterone and oestradiol on plasma progesterone concentrations and follicle wave dynamics in anovulatory anoestrous postpartum dairy cattle. Anim Reprod Sci 2004;84:303–14.

[12] Carvalho JB, Carvalho NA, Reis EL, Nichi M, Souza AH, Baruselli PS. Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus x Bos taurus, and Bos taurus heifers. Theriogenology 2008;69:167–75.

[13] Shaham-Albalancy A, Rosenberg M, Folman Y, Graber Y, Meidan R, Wolfenson D. Two methods of inducing low plasma progesterone concentrations have different effects on dominant follicles in cows. J Dairy Sci 2000;83:2771–8.

[14] Sanchez T, Wehrman ME, Kojima FN, Cupp AS, Bergfeld EG, Peters KE, et al. Dosage of the synthetic progestin, norgestomet, influences luteinizing hormone pulse frequency and endogenous secretion of 17 beta-estradiol in heifers. Biol Reprod 1995;52:464–9.

[15] Anderson LH, McDowell CM, Day ML. Progestin-induced puberty and secretion of luteinizing hormone in heifers. Biol Reprod 1996;54:1025–31.

[16] Adams GP, Matteri RL, Ginther OJ. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. J Reprod Fertil 1992;96:627–40.

[17] Leibfried-Rutledge ML, Critser ES, Eyestone WH, Northey DL, First NL. Development potential of bovine oocytes matured in vitro or in vivo. Biol Reprod 1987;36:376–83.

[18] Blondin P, Sirard MA. Oocyte and follicular morphology as determining characteristics for developmental competence in bovine oocytes. Mol Reprod Dev 1995;41:54–62.

[19] Pfeifer LF, Schneider A, Castilho EM, Luz EM, Ataide PF, Dionello NJ, et al. Effect of the exogenous progesterone on the follicular development and the in vitro embryo production in oocyte donors beef cows. Acta Scientiae Veterinarieae 2005;33:184.

[20] Rahe CH, Owens RE, Fleeger JL, Newton HJ, Harms PG. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinology 1980;107:498–503.

[21] Mihm M, Curran N, Hyttel P, Knight PG, Boland MP, Roche JF. Effect of dominant follicle persistence on follicular fluid oestradiol and inhibin and on oocyte maturation in heifers. J Reprod Fertil 1999;116:293–304.

[22] Revah I, Butler WR. Prolonged dominance of follicles and reduced viability of bovine oocytes. J Reprod Fertil 1996;106:39–47.

[23] Perry GA, Smith MF, Roberts AJ, MacNeil MD, Geary TW. Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. J Anim Sci 2007;85:684–9.

[24] Wettemann RP, Hill GM, Boyd ME, Spitzer JC, Forrest DW, Beal WE. Reproductive performance of postpartum beef cows after short-term calf separation and dietary energy and protein supplementation. Theriogenology 1986;26:433–43.

[25] Rogan D, Martinez MF, Bo GA, Chesta P, Feresin F, Mapletoft RJ. Progesterone release patterns from Cue-Mate in comparison to other intravaginal progesterone-releasing devices in lacting dairy cows. Reprod Fertil Dev 2007;19(1):126–7.

[26] Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil 1989;87:223–30.

[27] Martinez MF, Kastelic JP, Bo GA, Caccia M, Mapletoft RJ. Effects of oestradiol and some of its esters on gonadotrophin release and ovarian follicular dynamics in CIDR-treated beef cattle. Anim Reprod Sci 2005;86:37–52.

[28] Honaramooz A, Chandolia RK, Beard AP, Rawlings NC. Opioidergic, dopaminergic and adrenergic regulation of LH secretion in prepubertal heifers. J Reprod Fertil 2000;119:207–15.

[29] Purup S, Sejrsen K, Foldager J, Akers RM. Effect of exogenous bovine growth hormone and ovariectomy on prepubertal mammary growth, serum hormones and acute in-vitro proliferative response of mammary explants from Holstein heifers. J Endocrinol 1993;139:19–26.

[30] Singh J, Pierson RA, Adams GP. Ultrasound image attributes of bovine ovarian follicles and endocrine and functional correlates. J Reprod Fertil 1998;112:19–29.

[31] Rawlings NC, Jeffcoate IA, Rieger DL. The influence of estradiol-17beta and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized ewe. Theriogenology 1984;22:473–88.

[32] Schillo KK, Green MA, Hayes SH. Effects of adrenalectomy on photoperiod-induced changes in release of luteinizing hormone and prolactin in ovariectomized ewes. J Reprod Fertil 1988;83:431–8.

[33] Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 1998;76:1216–31.

[34] Martinez MF, Kastelic JP, Adams GP, Mapletoft RJ. The use of GnRH or estradiol to facilitate fixed-time insemination in an MGA-based synchronization regimen in beef cattle. Anim Reprod Sci 2001;67:221–9.

[35] Lucy MC, Billings HJ, Butler WR, Ehnis LR, Fields MJ, Kesler DJ, et al. Efficacy of an intravaginal progesterone insert and an injection of PGF2alpha for synchronizing estrus and shortening the interval to pregnancy in postpartum beef cows, peripubertal beef heifers, and dairy heifers. J Anim Sci 2001;79:982–95.

[36] Baruselli PS, Reis EL, Marques MO, Nasser LF, Bo GA. The use of hormonal treatments to improve reproductive performance of anestrous beef cattle in tropical climates. Anim Reprod Sci 2004;82–83:479–86.

[37] Colazo MG, Kastelic JP, Martinez MF, Whittaker PR, Wilde R, Ambrose JD, et al. Fertility following fixed-time AI in CIDR-treated beef heifers given GnRH or estradiol cypionate and fed diets supplemented with flax seed or sunflower seed. Theriogenology 2004;61:1115–24.

[38] Kasimanickam R, Collins JC, Wuenschell J, Currin JC, Hall JB, Whittier DW. Effect of timing of prostaglandin administration, controlled internal drug release removal and gonadotropin releasing hormone administration on pregnancy rate in fixed-time AI protocols in crossbred Angus cows. Theriogenology 2006;66:166–72.

[39] Schafer DJ, Bader JF, Meyer JP, Haden JK, Ellersieck MR, Lucy MC, et al. Comparison of progestin-based protocols to synchronize estrus and ovulation before fixed-time artificial insemination in postpartum beef cows. J Anim Sci 2007;85:1940–5.

[40] Peeler ID, Nebel RL, Pearson RE, Swecker WS, Garcia A. Pregnancy rates after timed AI of heifers following removal of intravaginal progesterone inserts. J Dairy Sci 2004;87:2868–73.

[41] Torres-Junior J, Sa Filho M, Gimenes L, Madureira E, Baruselli PS. Effect of PGF injected on the beggining of the Norgestomed treatment on the follicular dynamics of the nelore heifers (bos indicus). Acta Scientiae Veterinarieae 2005;33:262.

[42] Xu Z, Garverick HA, Smith GW, Smith MF, Hamilton SA, Youngquist RS. Expression of follicle-stimulating hormone and luteinizing hormone receptor messenger ribonucleic acids in bovine follicles during the first follicular wave. Biol Reprod 1995;53:951–7.

[43] Roberson MS, Wolfe MW, Stumpf TT, Kittok RJ, Kinder JE. Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. Biol Reprod 1989;41:997–1003.

[44] Stegner JE, Kojima FN, Bader JF, Lucy MC, Ellersieck MR, Smith MF, Patterson DJ. Follicular dynamics and steroid profiles in cows during and after treatment with progestin-based protocols for synchronization of estrus. J Anim Sci 2004;82:1022–8.

[45] Mihm M, Crowe MA, Knight PG, Austin EJ. Follicle wave growth in cattle. Reprod Domest Anim 2002;37:191–200.

[46] Bao B, Garverick HA, Smith GW, Smith MF, Salfen BE, Youngquist RS. Changes in messenger ribonucleic acid encoding luteinizing hormone receptor, cytochrome P450-side chain cleavage, and aromatase are associated with recruitment and selection of bovine ovarian follicles. Biol Reprod 1997;56:1158–68.

[47] Evans AC, Fortune JE. Selection of the dominant follicle in cattle occurs in the absence of differences in the expression of messenger ribonucleic acid for gonadotropin receptors. Endocrinology 1997;138:2963–71.

[48] Ireland JJ, Roche JF. Development of nonovulatory antral follicles in heifers: changes in steroids in follicular fluid and receptors for gonadotropins. Endocrinology 1983;112:150–6.

[49] Mann GE, Lamming GE, Robinson RS, Wathes DC. The regulation of interferon-tau production and uterine hormone receptors during early pregnancy. J Reprod Fertil Suppl 1999;54:317–28.

[50] Sá Filho MF, Penteado L, Reis EL, Gimenes LU, Baruselli PS. Effect of the cyclicity and eCG treatment on follicular dynamics and on the conception rate in nelore heifers treated with norgestomed ear implant and estradiol benzoate. Acta Scientiae Veterinarieae 2005;33:265.

[51] Small JA, Colazo MG, Kastelic JP, Mapletoft RJ. Effects of progesterone presynchronization and eCG on pregnancy rates to GnRH-based, timed-AI in beef cattle. Theriogenology 2009;71:698–706.

[52] Bó GA, Cutaia L, Chesta P, Moreno D. The use of eCG to increase pregnancy rates in postpartum beef cows following treatment with progesterone vaginal devices and estradiol benzoate and fixed time AI. Reprod Fertil Dev 2004;16:127.

[53] Cutaia L, Tribulo R, Moreno D, Bó GA. Pregnancy rates in lacting beef cows treated with progesterone releasing devices, estradiol benzoate and equine chorionic gonadotropin (eCG). Theriogenology 2003;59:216.

[54] McNatty KP, Smith DM, Makris A, Osathanondh R, Ryan KJ. The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the population of granulosa cells, and the status of the oocyte in vivo and in vitro. J Clin Endocrinol Metab 1979;49:851–60.

[55] Utt MD, Jousan FD, Beal WE. The effects of varying the interval from follicular wave emergence to progestin withdrawal on follicular dynamics and the synchrony of estrus in beef cattle. J Anim Sci 2003;81:1562–7.

[56] Kim UH, Suh GH, Hur TY, Kang SJ, Kang HG, Park SB, et al. Comparison of two types of CIDR-based timed artificial

insemination protocols for repeat breeder dairy cows. J Reprod Dev 2007;53:639–45.

[57] Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J Dairy Sci 2004;87:905–20.

[58] Kojima FN, Chenault JR, Wehrman ME, Bergfeld EG, Cupp AS, Werth LA, et al. Melengestrol acetate at greater doses than typically used for estrous synchrony in bovine females does not mimic endogenous progesterone in regulation of secretion of luteinizing hormone and 17 beta-estradiol. Biol Reprod 1995;52:455–63.

Available online at www.sciencedirect.com

ScienceDirect

Theriogenology

Theriogenology 72 (2009) 1054–1064

www.theriojournal.com

Effects of exogenous progesterone and cloprostenol on ovarian follicular development and first ovulation in prepubertal heifers

L.F.M. Pfeifer [a,b], L.G. Siqueira [a], R.J. Mapletoft [a], J.P. Kastelic [c], G.P. Adams [a], M.G. Colazo [d], J. Singh [a,*]

[a] *University of Saskatchewan, Saskatoon, SK, Canada*
[b] *Department of Animal Science, Universidade Federal de Pelotas, Pelotas, RS, Brazil*
[c] *Agriculture and Agri-Food Canada, Lethbridge, AB, Canada*
[d] *Alberta Agriculture and Rural Development, Edmonton, AB, Canada*

Received 4 March 2009; received in revised form 26 June 2009; accepted 28 June 2009

Abstract

The objective of this study was to determine the effects of progesterone and cloprostenol (a $PGF_{2\alpha}$ analogue) on ovarian follicular development and ovulation in prepubertal heifers. In Experiment 1, crossbred Hereford heifers (*Bos taurus*; 10 to 12 mo old, 255 to 320 kg) were assigned randomly to three groups and given (1) an intravaginal progesterone-releasing insert (CIDR; P group, n = 13); (2) a CIDR plus 500 μg cloprostenol im ($PGF_{2\alpha}$ analogue) at CIDR removal (PPG group, n = 11); or (3) no treatment (control group, n = 14). The CIDR inserts were removed 5 d after follicular wave emergence. Progesterone-treated heifers (P and PPG groups) had a larger dominant follicle than that of the control group (P = 0.01). The percentage ovulating was highest in the PPG group (8 of 11, 73%), intermediate in the P group (4 of 13, 31%), and lowest in the control group (1 of 14, 7%; P < 0.02). In Experiment 2, 16 heifers (14 to 16 mo old, 300 to 330 kg) were designated to have follicular wave emergence synchronized with either a CIDR and 1 mg estradiol benzoate im (EP group, n = 8) on Day 0 (beginning of experiment) or by transvaginal ultrasound-guided ablation of all follicles ≥5 mm on Day 3 (FA group, n = 8). On Day 7, CIDRs were removed in the EP group, and all heifers received 500 μg cloprostenol im. Ovulation was detected in 6 of 8 heifers (75%) in both groups. In summary, the use of $PGF_{2\alpha}$ with or without exogenous progesterone treatment increased the percentage ovulating in heifers close to spontaneous puberty.
© 2009 Elsevier Inc. All rights reserved.

Keywords: Cloprostenol; Follicular development; Ovulation; Prepubertal heifers; Progesterone

1. Introduction

A wave-like pattern of ovarian follicular development has been shown to occur in heifer calves as early as 2 wk of age [1], and the maximum diameter of dominant follicles continued to increase through the peripubertal period to puberty [1,2]. The pattern of follicular wave development [3,4] and mechanisms controlling follicle growth in the late prepubertal period and during physiologic estrus cycles in heifers appeared to be similar [5]. However, in some studies, reproductive function continued to improve after the first ovulation. In that regard, the maximum diameter of dominant follicles in peripubertal heifers was smaller than that of dominant follicles in older heifers [5–7]. Peripheral

* Corresponding author. Tel.: +1 306 966 7410; fax: +1 306 966 7405.
E-mail address: jaswant.singh@usask.ca (J. Singh).

0093-691X/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2009.06.022

luteinizing hormone (LH) concentrations and LH pulse frequency increased just before puberty in heifers [8,9]; this pattern of LH secretion was considered a critical signal for the onset of puberty [5,10]. Furthermore, increased secretion of estradiol-17β by the dominant follicle near the onset of puberty was considered responsible for the decrease of negative feedback of this steroid on LH secretion before the onset of puberty [5].

Heifers that reach puberty early in the breeding season are more likely to become pregnant in their first breeding season [11,12]. In that regard, puberty can be hastened with exogenous progestins, which modulate LH pulse frequency [13]. Exogenous progesterone decreases the number of hypothalamic estradiol receptors, resulting in increased LH pulse frequency after cessation of progesterone treatment [13,14].

Although prostaglandins (and their analogues) are primarily used as luteolysins, they also affect ovulation, implantation, pregnancy maintenance, parturition, and postpartum physiology [15]. Prostaglandins have been used for estrus synchronization, either by themselves or in combination with progestins, estrogens, and gonadotropin-releasing hormone (GnRH) [15]. The effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on ovulation include increased pituitary responsiveness to GnRH, thereby augmenting LH release in postpartum cows and prepubertal heifers [16]. Furthermore, $PGF_{2\alpha}$ may enhance the effects of exogenous progesterone on the hypothalamus after progesterone withdrawal, thereby increasing pituitary responsiveness to GnRH [16] and inducing ovulation in ewes and cattle [15,17].

The objectives of the current study were to investigate the effects of (1) exogenous progesterone on dominant follicle growth and ovulation in prepubertal heifers; and (2) exogenous $PGF_{2\alpha}$ on ovulation, with or without pretreatment with exogenous progesterone. Two hypotheses were tested: (a) Exogenous progesterone, starting at or before wave emergence, will increase the growth rate of the dominant follicle, resulting in ovulation after progesterone withdrawal; (b) exogenous $PGF_{2\alpha}$ treatment at the end of the growth phase of the dominant follicle, with or without pretreatment with exogenous progesterone, will enhance the percentage ovulating in peripubertal heifers. Two experiments were conducted. Experiment 1 determined the effects of progesterone treatment, with or without $PGF_{2\alpha}$ at progesterone removal, on ovarian follicular development and ovulation in prepubertal heifers, whereas Experiment 2 determined the effectiveness of $PGF_{2\alpha}$, with or without exogenous progesterone, to induce the first ovulation in peripubertal heifers after synchronization of follicular wave emergence.

2. Materials and methods

2.1. Experiment 1

This study was conducted with 38 crossbred Hereford prepubertal heifers (*Bos taurus*; 10 to 12 mo of age, 255 to 320 kg body weight) maintained in outdoor pens at the University of Saskatchewan Goodale Research Farm, SK (52°03' N, 106°30' W). Heifers were fed barley silage and alfalfa hay, with ad libitum access to water, salt, and a mineral mixture. Transrectal ultrasonographic examinations (Aloka SSD-900, B-mode scanner with a 7.5 MHz linear transducer; Aloka, Tokyo, Japan) were done on two occasions 11 d apart to confirm the absence of a corpus luteum (CL; i.e., all heifers were prepubertal). The heifers were assigned randomly into three groups (Fig. 1A) and given (1) an intravaginal progesterone-releasing insert (CIDR; P group, n = 13); (2) a CIDR plus 500 μg im of a $PGF_{2\alpha}$ analogue, cloprostenol (Estrumate; Schering-Plough Animal Health, Pointe-Claire, QC, Canada) at CIDR removal (PPG group, n = 11); or (3) no treatment (control group, n = 14). A CIDR (1.9 g progesterone; Pfizer Animal Health, Montreal, QC, Canada) was given to each heifer in the progesterone-treated groups at random stages of the follicular wave, whereas heifers in the control group received no treatment. Heifers were examined daily by transrectal ultrasonography to monitor ovarian follicular dynamics and to detect ovulation. At each examination, a sketch of each ovary was made, and the diameter and location of all follicles ≥4 mm in diameter were recorded. The day of wave emergence, defined as the day when the dominant follicle of a wave was first recorded (diameter, 4 to 5 mm), was determined retrospectively and designated as Day 0. In progesterone-treated heifers, CIDRs were removed on Day 5. Ultrasonographic examinations were conducted daily from the beginning of the experiment to ovulation (all groups) or, in the absence of ovulation, up to 5 d after CIDR removal (P and PPG groups), or to detection of a dominant follicle ≥8 mm in diameter in the subsequent follicular wave (control group). Ovulation was defined as the disappearance of a previously identified follicle ≥8 mm in diameter [18]. In addition to ovarian ultrasonographic examinations, the uterus of each heifer was carefully examined by transrectal palpation and ultrasonography to identify signs of proestrus (i.e., increased uterine tone and intrauterine fluid) [19,20]. In heifers that ovulated, two ultrasonographic examinations were performed (7 and 10 d after ovulation) to determine CL diameter and detect premature luteolysis.

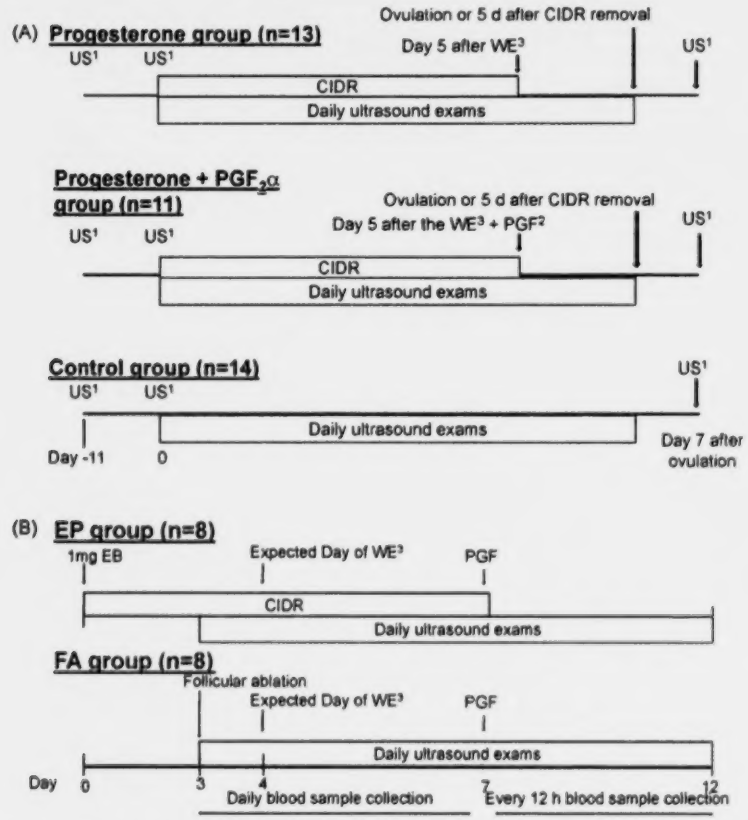


Fig. 1. (A) Timeline of treatments in Experiment 1. Prepubertal heifers were given an intravaginal progesterone-releasing device (CIDR; P group, n = 13); a CIDR plus 500 μg cloprostenol im ($PGF_{2\alpha}$ analogue) at CIDR removal (PPG group, n = 11); or no treatment (control group, n = 14). The CIDRs were removed 5 d after the wave emergence. (B) Timeline of treatments in Experiment 2. Prepubertal beef heifers received a CIDR insert containing 1.9 g progesterone and 1 mg estradiol benzoate (EB; EP group, n = 8) or were subjected to follicular ablation (FA group, n = 8). All heifers received 500 μg cloprostenol im ($PGF_{2\alpha}$ analogue) on Day 7, and CIDRs were removed in the EP group. [1]US, transrectal ultrasonography; [2]PGF, 500 μg cloprostenol ($PGF_{2\alpha}$ analogue); [3]WE, wave emergence.

Blood samples were collected by caudal venipuncture into heparinized 10-mL tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) just prior to CIDR insertion and then once daily until 5 d after CIDR removal (P and PPG groups), or until detection of an 8-mm dominant follicle in the subsequent follicular wave (control group). Blood samples were centrifuged (1500 × *g* for 15 min), and plasma was harvested and stored at −20 °C.

2.2. Experiment 2

Twenty-four crossbred Hereford heifers (14 to 16 mo old, 300 to 330 kg body weight) were examined ultrasonographically on two occasions, 7 d apart, to confirm the absence of a CL. Heifers in which a CL was not detected at either ultrasonographic examination were used (n = 16). Heifers were housed, fed, and handled as in Experiment 1. Peripubertal heifers were randomly assigned to one of two treatment groups (n = 8/group; Fig. 1B) to induce synchronous wave emergence with either progesterone-estradiol treatment (EP group) or by follicle aspiration (FA group). Heifers in the EP group received a CIDR insert and 1 mg estradiol benzoate (Sigma Chemical Company, St. Louis, MO, USA) in 2 mL canola oil on Day 0 (start of the experiment). The CIDR inserts were removed on Day 7. Heifers in the FA group were subjected to ablation of all follicles >5 mm in diameter [21] on Day 3. Emergence of a new follicular wave was expected on Day 4 in both groups [21]. All heifers received 500 μg cloprostenol im on Day 7. Transrectal ultrasonography

was done as in Experiment 1, once daily from Day 3 (1 d before the expected follicular wave emergence) to ovulation or to Day 12 (in the absence of ovulation).

Blood samples were collected and processed as in Experiment 1. The samples used to measure plasma LH concentrations were collected once daily from Days 3 to 7 and then every 12 h until ovulation, or to Day 12. The samples used to measure plasma concentrations of progesterone and estradiol were collected on Days 3 (12 h after ablation in the FA group), 5, and 7.

2.3. Radioimmunoassays

Plasma progesterone concentrations were assayed with a solid-phase radioimmunoassay [22] with a minimum detection limit of 0.1 ng/mL. Progesterone was analyzed after extraction with 3 mL hexane from 200-μL aliquots of plasma. In Experiment 1, the intra-assay CVs were 5.7% (1.6 ng/mL) and 5.7% (16.5 ng/mL) for low and high progesterone reference samples, respectively. In Experiment 2, the intra-assay CVs were 10.0% (0.36 ng/mL) and 8.6% (1.11 ng/mL) for low and high progesterone reference samples, respectively.

Plasma estradiol concentrations were measured with a modified human double-antibody RIA Kit (DPC Coat-a-count; Diagnostic Products Corporation, Los Angeles, CA, USA [23]) using a procedure described by Singh et al. [24]. The sensitivity of the assay was 0.5 pg/mL, and the intra-assay CVs were 4.2% (21.1 pg/mL) and 2.8% (89.1 pg/mL) for low and high estradiol concentrations.

Plasma LH concentrations were determined by double-antibody radioimmunoassay [25] and expressed in terms of NIDDK-bLH-4. The minimum detection limit was 0.06 ng/mL, with a standard curve ranging from 0.06 to 8 ng/mL. In Experiment 1, intra-assay CVs were 7.6% (0.65 ng/mL) and 8.3% (2.05 ng/mL), for low and high LH concentrations, respectively. In Experiment 2, the intra-assay coefficients of variation were 3.9% (0.95 ng/mL) and 6.0% (2.4 ng/mL) for low and high LH concentrations, respectively. For each hormone, all samples for each experiment were included in a single assay.

2.4. Statistical analyses

Single-point measures (e.g., maximum diameter of the ovulatory follicle, growth rate of the ovulatory follicle, CL diameter 7 d after ovulation, day of ovulation, and progesterone concentrations at the beginning of treatment) were compared among groups by one-way ANOVA. The follicular growth rate was measured according Ginther et al. [7]. Percentage ovulating and the proportion of heifers showing signs of proestrus were compared by chi-square analysis or by Fisher's exact test (if the "expected value" was <5). Pregnancy rate was also analyzed by chi-square, after combining groups P and PPG versus control. Analyses involving repeated measures over time (e.g., follicle diameter, plasma LH concentrations, and plasma concentrations of progesterone and estradiol on Days 3, 5, and 7) were compared among groups, and also between heifers that ovulated or did not ovulate, by the MIXED procedure, using SAS 9.0 (SAS Institute Inc., Cary, NC, USA [26]) for repeated measures to evaluate the main effects of treatment and time (days), and their interactions [27]. When interactions were significant ($P < 0.05$), a least square difference test was used to detect treatment effects at each time. As two treatment groups received progesterone treatment, the dominant follicle growth data of the P and PPG groups were combined and compared with that of control groups using the MIXED procedure. In Experiment 2, data for LH concentration were aligned to the LH rises (highest LH concentration).

3. Results

3.1. Experiment 1

Percentage of heifers ovulating tended to be higher in the PPG group than in the P group ($P = 0.09$; Table 1). Overall, progesterone treatment (P and PPG groups combined) resulted in a higher ($P < 0.01$) incidence of ovulation (12 of 24, 50%) compared with that of the control group (1 of 14, 7.1%). Percentages of heifers ovulating and those with signs of proestrus, as well as characteristics of the follicular wave are summarized in Table 1.

Ovarian follicular growth profile are summarized (Fig. 2). In heifers that ovulated versus those that did not ovulate, growth rate of the dominant follicle was faster (1.1 ± 0.05 mm/d and 0.96 ± 0.02 mm/d, respectively) and maximum diameter was larger (12.9 ± 0.27 mm and 11.8 ± 0.20 mm; $P < 0.002$ for each). The CL diameter 7 d after ovulation did not differ between the P and PPG groups (19.5 ± 2.5 mm and 22.9 ± 1.3 mm, respectively) and seemed to be larger (formal statistical analysis was not possible) than in the single control heifer that ovulated (11.0 mm). In the latter heifer, 10 d after ovulation, the CL was undetectable, indicating a short estrous cycle. However, none of the heifers in the P and PPG groups had a short estrous cycle after ovulation.

Table 1
Percentages of heifers ovulating and those with signs of proestrus, and mean (±SEM) characteristics of the follicular wave of prepubertal heifers treated with a CIDR (P group), a CIDR plus $PGF_{2\alpha}$ (PPG group), or no treatment (control group) in Experiment 1.

	P group	PPG group	Control group	P value
Percentage ovulating	4/13 (30.8%)[a]	8/11 (72.7%)[a]	1/14 (7.1%)[b]	0.02
Proestrus signs	8/13 (61.5%)[a]	9/11 (81.8%)[a]	2/14 (14.3%)[b]	0.02
Interval from wave emergence to ovulation (d)	8.0 ± 0.0^{a}	8.5 ± 0.25^{ab}	10.0^{b}	0.03
Maximum diameter of dominant follicle (mm)	12.5 ± 0.25^{a}	12.7 ± 0.34^{a}	11.5 ± 0.25^{b}	<0.01
Maximum diameter of dominant anovulatory follicle (mm)	12.5 ± 0.26^{a}	11.3 ± 0.16^{b}	11.3 ± 0.29^{b}	0.017
Growth rate of all dominant follicles (mm/d)	1.1 ± 0.04^{a}	1.1 ± 0.06^{a}	0.9 ± 0.02^{b}	<0.03

*Groups were compared by Fisher's exact tests.
[a,b]Within a row, means without a common superscript differed ($P < 0.05$).

There was no significant difference among groups in progesterone concentrations on Day 0. As expected, heifers in the P and PPG groups had higher plasma progesterone concentrations than that of heifers in the control group 5 d after follicular wave emergence (due to CIDR treatment) and 7 d after ovulation, due to the presence of a CL (Fig. 3). Plasma LH concentrations from 5 d after follicle wave emergence to Day 9 are shown in Figure 4. There was no treatment effect, but there tended to be an effect of day (Fig. 4A). Considering only heifers that ovulated, there was an effect of Day and tendency for a treatment-by-day interaction ($P = 0.07$; Fig. 4B).

3.2. Experiment 2

Follicular wave emergence occurred 0.75 d earlier ($P < 0.03$) in the EP group (two heifers on Days 2 and 3 and four on Day 4) than in the FA group (all heifers had follicular wave emergence on Day 4; i.e., 1 d after follicle ablation).

There was no significant difference between the two treatment groups for the proportion of heifers that ovulated after $PGF_{2\alpha}$ treatment (6 of 8; 75% for both groups); the interval from $PGF_{2\alpha}$ treatment to ovulation (Fig. 5A); the diameter of the dominant follicle at selection and its growth rate; the duration of the ovulatory wave (from wave emergence to ovulation); and the diameter of the ovulatory follicle prior to ovulation. Overall mean ± SEM for both groups were 8.1 ± 0.56 mm for diameter of the dominant follicle at selection, 1.7 ± 0.09 mm/d for growth rate of the dominant follicle, 7.6 ± 0.36 d for duration of the ovulatory wave, and 13.4 ± 0.55 mm for largest diameter of the ovulatory follicle.

There was a day effect for dominant follicle development (Fig. 5A). When growth of the dominant follicle was compared between heifers that did or did not ovulate, there was a tendency for effects of treatment and day (Fig. 5C).

Plasma LH concentrations before the LH rises were affected by treatment and day; heifers in the FA group had higher mean LH concentrations than that of heifers in the EP group (0.6 ± 0.09 and 0.3 ± 0.08 ng/mL; Fig. 5B). When the mean LH concentration was compared between heifers that did or did not ovulate, the former had higher mean LH concentration than that of the latter after the LH rises ($P = 0.033$; Fig. 5D). There was an effect of treatment and day on plasma estradiol concentrations (Fig. 6A). Although heifers in the EP group had higher estradiol concentrations than that of the FA group on Days 3 and 5, there was no difference on Day 7 (the time of CIDR removal in the EP group). There were treatment and day effects and a treatment-by-day interaction on plasma progesterone concentrations on Days 3, 5, and 7 (Fig. 6B).

4. Discussion

The higher incidence of ovulation in heifers given progesterone compared with that of control heifers was similar to that reported previously [13,28,29]. However, the percentage ovulating tended to be higher in the prepubetal heifers that were treated with $PGF_{2\alpha}$ and progesterone versus those given only progesterone in Experiment 1, and an equal number of heifers ovulated after $PGF_{2\alpha}$ treatment in Experiment 2, regardless of whether they were given exogenous progesterone. Therefore, our hypothesis that exogenous $PGF_{2\alpha}$ treatment at the end of growth phase of the dominant follicle, with or without pretreatment with exogenous progesterone, will enhance percentage ovulating in prepubertal heifers was supported. We inferred that $PGF_{2\alpha}$ promoted ovulation by mechanisms other than luteolysis. Weens et al. [15] suggested a direct effect of $PGF_{2\alpha}$ on the anterior pituitary; apparently increasing

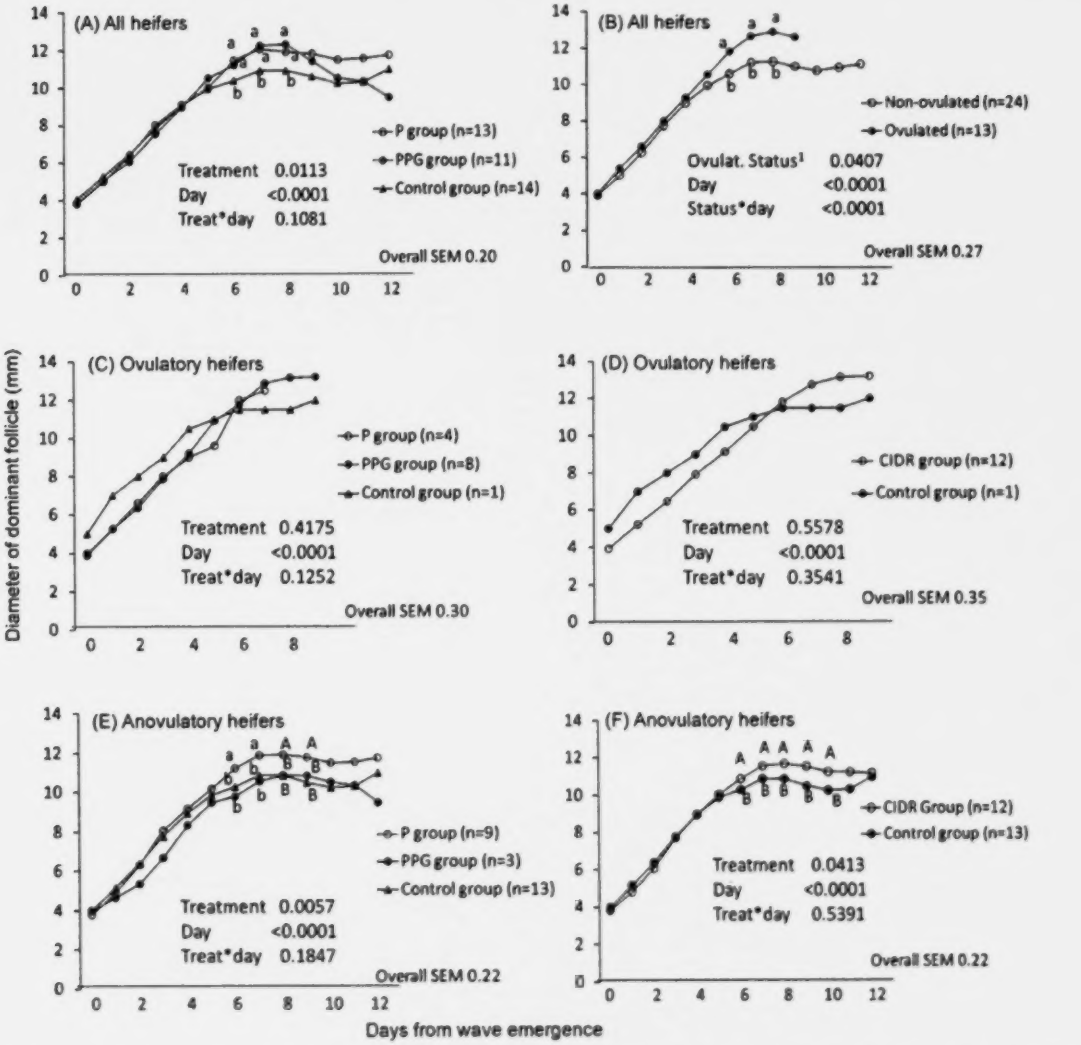


Fig. 2. Pattern of dominant ovarian follicle growth in all prepubertal heifers used in Experiment 1. (A, C, E) Heifers in the P (-○-) and PPG (-●-) groups received a CIDR, and heifers in the PPG group received 500 μg cloprostenol on the day of CIDR removal (5 d after follicle wave emergence), whereas the control group (-▲-) received no treatment. (B) Pattern of dominant ovarian follicle growth in prepubertal heifers comparing heifers that ovulated (-●-) with those that did not ovulate (-○-), regardless of treatment group. (D, F) The P and PPG groups were combined (-○-) and compared with the controls (-●-). [a,b]Within a day, values without a common superscript differed ($P < 0.05$). [A,B]Within a day, values without a common superscript tended to differ ($P < 0.1$).

pituitary responsiveness to GnRH and thereby enhancing the release of LH [16]. Although the combination of progesterone and $PGF_{2\alpha}$ appeared to act synergistically, $PGF_{2\alpha}$ after follicle ablation seemed just as efficacious (Experiment 2). Progesterone may have reduced the negative feedback of estradiol on the hypothalamus by reducing the number of estradiol receptors [8], whereas $PGF_{2\alpha}$ may have increased the responsiveness of the pituitary to GnRH [16]. Furthermore, the groups treated with prostaglandin showed an increasing of LH concentrations earlier than that in control heifers. Although heifers in Experiment 2 were prepubertal, they were older and heavier than heifers in Experiment 1. The high percentage ovulating in the FA group could be an effect of $PGF_{2\alpha}$ in association with follicular ablation; the latter could have affected the

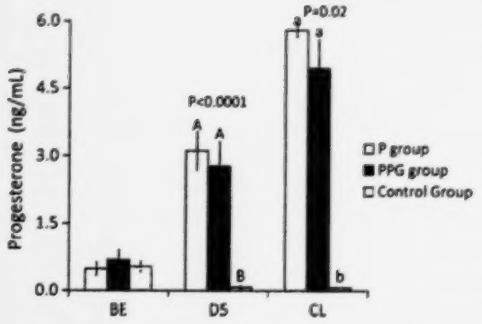


Fig. 3. Mean (±SEM) plasma progesterone concentrations in prepubertal heifers that received a CIDR (P group), a CIDR plus 500 μg cloprostenol (PPG group), or no treatment (control group). BE, beginning of the experiment; D5, 5 d after follicular wave emergence; CL, 7 d after ovulation. Superscripts show the difference among groups within each day. On D5, the P and PPG groups were different than control group ($P < 0.0001$) as well as on Day of CL ($P < 0.02$).

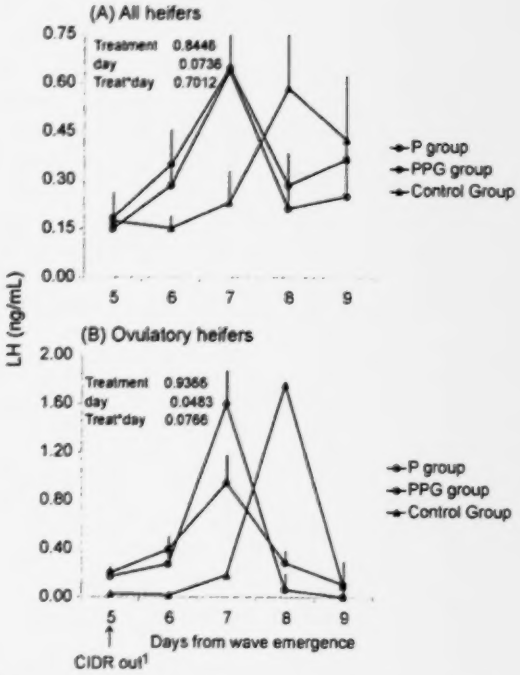


Fig. 4. (A) Mean (±SEM) LH concentration in all prepubertal heifers that received CIDR (P group; -○-), CIDR plus 500 μg cloprostenol (PPG group; -●-), or no treatment (control group; -▲-). (B) Mean (±SEM) LH concentration in ovulated prepubertal heifers. [1]CIDR out indicates day of CIDR removal in the P and PPG groups.

feedback mechanisms of estradiol that controls the onset of puberty. In that regard, the hypothalamo-pituitary axis is less affected by the negative feedback of estradiol on the GnRH secretion in cyclic compared with prepubertal cattle [8].

Treatment with progesterone plus $PGF_{2\alpha}$ (PPG group in Experiment 1 and EP group in Experiment 2) resulted in similar percentages of heifers ovulating, clearly demonstrating the ability to ovulate (hypothalamo-pituitary axis responded to treatment) in heifers as young as 10 mo of age. This effect was especially interesting in the PPG group, as these heifers were approximately 4 mo younger than those in the EP group. Within progesterone-treated heifers in Experiment 1, 5 of 12 that did not ovulate had signs of proestrus, suggesting that, after progesterone treatment, they had increased estradiol concentrations [5,30], which induced signs of proestrus [20].

Estradiol decreased the LH pulse amplitude in sheep and cattle [22], and estradiol per se [31,32], or in combination with progesterone [33], suppressed follicle-stimulating hormone (FSH). Thus, the sudden removal of the negative feedback of estradiol in the FA group through the removal of the follicular fluid could have augmented secretion of LH, in addition to the FSH surge that was expected to precede the new follicular wave [34,35]. Furthermore, reduced estradiol concentrations after follicle ablation (Fig. 6) in association with the $PGF_{2\alpha}$ treatment may have increased pituitary responsiveness to endogenous GnRH [15,16]. Conversely, it is plausible that follicular ablation may have resulted in partial luteinization to produce small amounts of endogenous progesterone. In this regard, plasma progesterone concentrations in the FA group showed a slight increase (~0.6 ng/mL) but were not significantly different 4 d after ablation compared with 2 d after ablation.

The positive effect of progesterone on the diameter of the dominant follicle in prepubertal heifers has been previously reported [13,36]. The faster rate of growth of the dominant follicle in progesterone-treated heifers compared with that in controls in Experiment 1 may have been a consequence of the suppressive effect of progesterone on the estradiol negative feedback on the hypothalamus. Once the number of estradiol receptors in the hypothalamus are reduced, follicular steroidogenesis increases as a result of an increase in gonadotropin (FSH and LH) release [5,34,37], increasing the rate of growth and diameter of the dominant follicle. The effect of progesterone on the hypothalamus was presumably exerted when the CIDR was in place. Similar effects were not observed in Experiment 2; progesterone-treated and nontreated heifers had the same rate of dominant follicular growth. Furthermore, these heifers were also near puberty, with a hypothalamus that was sufficiently

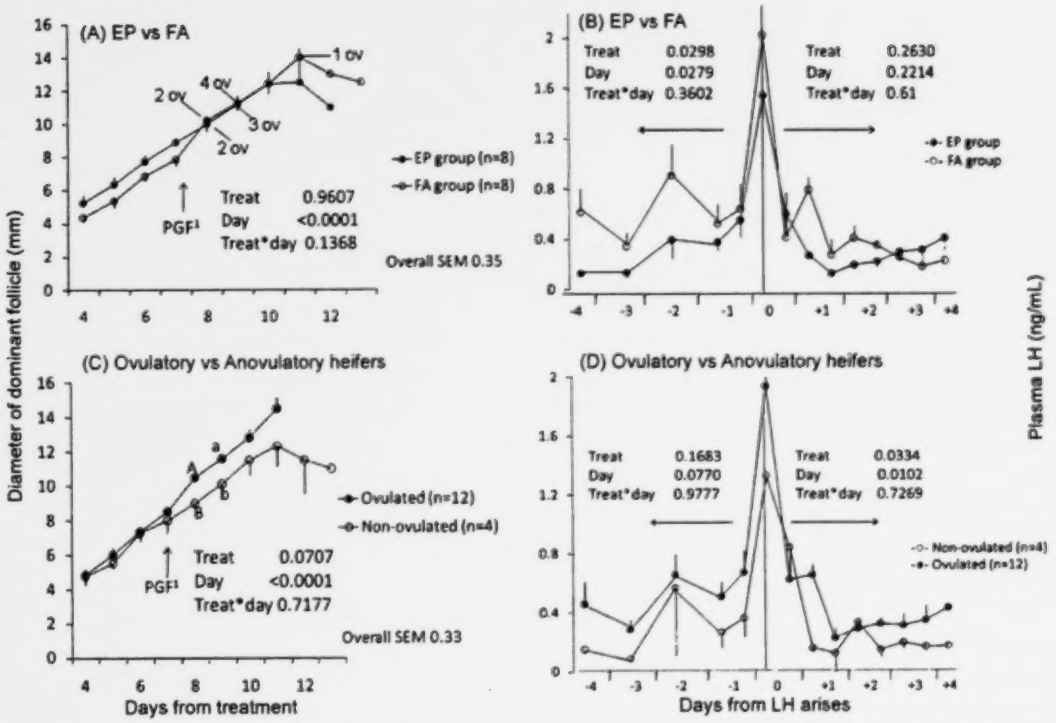


Fig. 5. Pattern of (A, C) dominant follicle growth and (B, D) LH secretion in Experiment 2. (A) Pattern of the dominant ovarian follicle growth in peripubertal heifers treated with CIDR and estradiol benzoate (EP group, -●-) and no progesterone (FA group, -○-). (B) Mean LH concentrations before and after the LH rises in CIDR-treated (EP group, - ○-) and nontreated (FA group,- ●-) heifers. Day 4 = 1 d after follicle ablation in FA group. (C) Comparison of the pattern of dominant ovarian follicle growth in the peripubertal heifers that ovulated versus those that did not ovulate. The day of follicular wave emergency was normalized to the mean for each treatment group. (D) Mean LH concentrations in ovulated and nonovulated peripubertal heifers. The horizontal bars on (B) and (D) indicate the repeated measures results before and after LH rises. Data for LH results were normalized for each heifer according to its rises. [a,b]Lowercase letters within the same day show difference ($P < 0.05$). [A,B]Uppercase letters within the same day show tendency ($P < 0.1$). [1]500 μg cloprostenol im.

mature to mask the effects of progesterone on follicular growth.

Although the effect of progesterone on follicular dynamics in prepubertal heifers has been reported [13,36], this is apparently the first report of a positive effect of progesterone on follicular growth in prepubertal heifers during a spontaneous (noninduced) follicular wave. It has been reported that the pattern of follicular growth during the late prepubertal period and first cycles after the onset of puberty was similar [4,5,7]. In the current study, the dominant follicles of the ovulatory waves were larger (similar to cycling heifers) than those of nonovulatory waves (similar to prepubertal period). However, dominant follicle growth profiles in progesterone-treated heifers that did not ovulate were larger than that in heifers not treated with progesterone (Experiment 1), further evidence of the positive effect of progesterone on follicular development in prepubertal heifers. In contrast, there was no difference among groups when the development of the ovulatory wave was compared (Experiment 1); however, only one heifer ovulated in the control group in Experiment 1, making it impossible to draw inferences regarding this group. Notwithstanding, follicular growth was clearly different between ovulatory and nonovulatory waves. Progesterone enhanced follicular growth in prepubertal heifers, and the outcome of the follicular wave (ovulatory or not) also was associated with the growth rate of the dominant follicle.

Progestin treatment increased follicular development in prepubertal heifers, even when LH secretion was not influenced [36]. Anderson et al. [13] suggested that exogenous progestin could lead to the development of a single large follicle. A similar effect was observed in anestrous postpartum cows treated with a progestin [38]. Conversely, low progesterone concentrations in

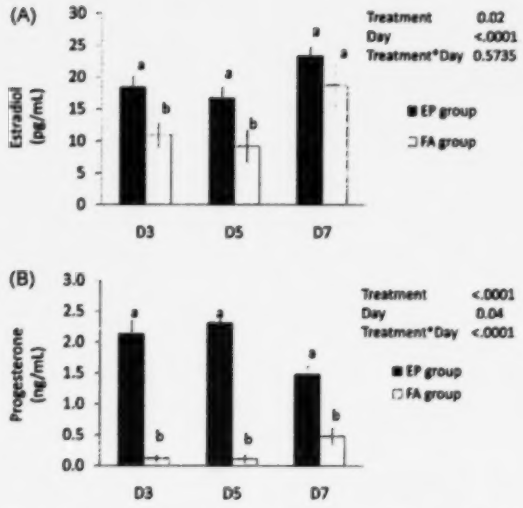


Fig. 6. (A) Mean (±SEM) plasma estradiol concentrations and (B) mean (±SEM) plasma progesterone concentrations in CIDR-treated (EP group) and nontreated (FA group) heifers in Experiment 2. Estradiol concentrations differed between groups on Day (D) 3 and on D5, but not at the end of the treatment (D7). Progesterone concentrations were higher in the EP group ($P < 0.0001$) on D3, D5, and D7.

cycling cattle after progestin treatment resulted in development of a large follicle compared with those under physiologic progesterone concentrations [39]. Apparently, low progesterone plasma concentrations in prepubertal heifers induce a higher LH secretion [40]; this effect could increase the diameter and growth rate of the dominant follicle in these heifers.

As expected, all heifers in the current experiment were considered prepubertal; no luteal tissue was detected ultrasonographically, and they had progesterone concentrations <1 ng/mL at the beginning of the study [13]. Furthermore, CIDR-treated groups had higher progesterone concentrations than those of the control group at CIDR removal (5 d after follicle wave emergence) [18]. However, it was noteworthy that progesterone concentrations 7 d after ovulation in Experiment 1 were higher in progesterone-treated groups than those in the control group (only one ovulation), which had progesterone concentration lower than expected for a 7-d-old CL [41]. Moreover, the CL in this heifer had apparently regressed by 10 d after ovulation, suggesting that a short estrous cycle had occurred. An increased incidence of short estrous cycles after the first pubertal ovulation has been reported [42,43]. Evans et al. [5] reported that all prepubertal heifers had a CL with a short life span after the first ovulation. In contrast, in the current study, the only heifer with a CL with a short life span was from the control group. Consistent with previous reports, CIDR treatment overcame the problem of short cycles after the first ovulation in heifers from the P and PPG groups [5,28]. Therefore, the CL (7 d after ovulation) was larger and produced more progesterone in heifers given exogenous progesterone compared with that in controls, resulting in normal-duration estrous cycles after the first ovulation. Unfortunately, CL development in Experiment 2 was not monitored after ovulation, so it was not possible to determine if the high percentage ovulating observed in the FA group resulted in corpora lutea with a normal life span.

An important objective of the beef cattle industry is to improve production efficiency by decreasing age at puberty. It was noteworthy that the EP treatment and $PGF_{2\alpha}$ treatment can be given irrespective of pubertal status and stage of follicular development and will induce ovulation for fixed-time insemination in both pubertal and prepubertal heifers. In that regard, in cycling versus prepubertal heifers, pregnancy rates were 54.2% versus 50.0% ($P < 0.21$) in CIDR-treated heifers and were 58.4% versus 46.4% ($P < 0.01$) in melengestrol acetate (MGA) treated heifers [44].

In summary, progesterone treatment enhanced dominant follicle development after spontaneous wave emergence in prepubertal heifers, and the use of $PGF_{2\alpha}$ in association with exogenous progesterone treatment hastened first ovulation. Giving $PGF_{2\alpha}$ without exogenous progesterone also induced ovulation after wave synchronization in heifers close to spontaneous puberty, providing a potential method to hasten the onset of puberty. It is unknown whether exogenous $PGF_{2\alpha}$ (without progesterone) will overcome the occurrence of short cycles after the first ovulation.

Acknowledgments

This research was supported by grants from the Saskatchewan Agriculture Development Fund and the Natural Sciences and Engineering Research Council of Canada. Luiz Pfeifer was supported by a CAPES scholarship from the Education Ministry of Brazil. The authors thank Pfizer Animal Health for CIDR inserts and Schering-Plough Animal Health for Estrumate, as well as personnel from the Goodale farm for providing heifers and technical assistance.

References

[1] Evans AC, Adams GP, Rawlings NC. Follicular and hormonal development in prepubertal heifers from 2 to 36 weeks of age. J Reprod Fertil 1994;102:463–70.

[2] Bergfeld EG, Kojima FN, Cupp AS, Wehrman ME, Peters KE, Garcia-Winder M, Kinder JE. Ovarian follicular development in prepubertal heifers is influenced by level of dietary energy intake. Biol Reprod 1994;51:1051–7.
[3] Adams GP, Evans AC, Rawlings NC. Follicular waves and circulating gonadotrophins in 8-month-old prepubertal heifers. J Reprod Fertil 1994;100:27–33.
[4] Sirois J, Fortune JE. Ovarian follicular dynamics during the estrous cycle in heifers monitored by real-time ultrasonography. Biol Reprod 1988;39:308–17.
[5] Evans AC, Adams GP, Rawlings NC. Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. J Reprod Fertil 1994;100:187–94.
[6] Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth of dominant follicles during the oestrous cycle of heifers. J Reprod Fertil 1988;83:663–71.
[7] Ginther OJ, Kastelic JP, Knopf L. Composition and characteristics of follicular waves during the bovine estrous cycle. Anim Reprod Sci 1989;20:187–200.
[8] Day ML, Imakawa K, Wolfe PL, Kittok RJ, Kinder JE. Endocrine mechanisms of puberty in heifers. Role of hypothalamo-pituitary estradiol receptors in the negative feedback of estradiol on luteinizing hormone secretion. Biol Reprod 1987;37: 1054–65.
[9] Kinder JE, Day ML, Kittok RJ. Endocrine regulation of puberty in cows and ewes. J Reprod Fertil Suppl 1987;34:167–86.
[10] Madgwick S, Evans AC, Beard AP. Treating heifers with GnRH from 4 to 8 weeks of age advanced growth and the age at puberty. Theriogenology 2005;63:2323–33.
[11] Yelich JV, Wettemann RP, Marston TT, Spicer LJ. Luteinizing hormone, growth hormone, insulin-like growth factor-I, insulin and metabolites before puberty in heifers fed to gain at two rates. Domest Anim Endocrinol 1996;13:325–38.
[12] Leismeister JL, Burfening PJ, Blackwell RL. Date of first calving in beef cows and subsequent calf production. J Anim Sci 1973;36:1–6.
[13] Anderson LH, McDowell CM, Day ML. Progestin-induced puberty and secretion of luteinizing hormone in heifers. Biol Reprod 1996;54:1025–31.
[14] Imwalle DB, Patterson DJ, Schillo KK. Effects of melengestrol acetate on onset of puberty, follicular growth, and patterns of luteinizing hormone secretion in beef heifers. Biol Reprod 1998;58:1432–6.
[15] Weems CW, Weems YS, Randel RD. Prostaglandins and reproduction in female farm animals. Vet J 2006;171:206–8.
[16] Randel RD, Lammoglia MA, Lewis AW, Neuendorff DA, Guthrie MJ. Exogenous PGF(2)alpha enhanced GnRH-induced LH release in postpartum cows. Theriogenology 1996;45: 643–54.
[17] Murdoch WJ, McCormick RJ. Mechanisms and physiological implications of leucocyte chemoattraction into periovulatory ovine follicles. J Reprod Fertil 1993;97:375–80.
[18] Martinez MF, Kastelic JP, Bo GA, Caccia M, Mapletoft RJ. Effects of oestradiol and some of its esters on gonadotrophin release and ovarian follicular dynamics in CIDR-treated beef cattle. Anim Reprod Sci 2005;86:37–52.
[19] Pierson RA, Ginther OJ. Ultrasonographyc appearance of the bovine uterus during the estrous cycle. J Am Vet Med Assoc 1987;190:995–1001.
[20] Senger PL. Reproduction cyclicity - the follicular phase. In: Senger PL, editor. Pathways to Pregnancy and Parturition. 2nd Edition. Current Conceptions, Inc.; 2003. pp. 164–87.
[21] Martinez MF, Adams GP, Kastelic JP, Bergfel DR, Mapletoft RJ. Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. Theriogenology 2000;54:757–69.
[22] Rawlings NC, Jeffcoate IA, Rieger DL. The influence of estradiol-17beta and progesterone on peripheral serum concentrations of luteinizing hormone and follicle stimulating hormone in the ovariectomized ewe. Theriogenology 1984;22:473–88.
[23] Purup S, Sejrsen K, Foldager J, Akers RM. Effect of exogenous bovine growth hormone and ovariectomy on prepubertal mammary growth, serum hormones and acute in-vitro proliferative response of mammary explants from Holstein heifers. J Endocrinol 1993;139:19–26.
[24] Singh J, Pierson RA, Adams GP. Ultrasound image attributes of bovine ovarian follicles and endocrine and functional correlates. J Reprod Fertil 1998;112:19–29.
[25] Honaramooz A, Chandolia RK, Beard AP, Rawlings NC. Opioidergic, dopaminergic and adrenergic regulation of LH secretion in prepubertal heifers. J Reprod Fertil 2000;119:207–15.
[26] SAS. Learning Edition 4.1 Entreprise Guide. SAS Institute Inc., 2006.
[27] Littell RC, Henry PR, Ammerman CB. Statistical analysis of repeated measures data using SAS procedures. J Anim Sci 1998;76:1216–31.
[28] Patterson DJ, Corah LR, Brethour JR. Response of prepubertal Bos taurus and Bos indicus x Bos taurus heifers to melengestrol acetate with or without gonadotropin-releasing hormone. Theriogenology 1990;33:661–8.
[29] Lucy MC, Billings HJ, Butler WR, Ehnis LR, Fields MJ, Kesler DJ, et al. Efficacy of an intravaginal progesterone insert and an injection of PGF2alpha for synchronizing estrus and shortening the interval to pregnancy in postpartum beef cows, peripubertal beef heifers, and dairy heifers. J Anim Sci 2001;79:982–95.
[30] Stegner JE, Kojima FN, Bader JF, Lucy MC, Ellersieck MR, Smith MF, Patterson DJ. Follicular dynamics and steroid profiles in cows during and after treatment with progestin-based protocols for synchronization of estrus. J Anim Sci 2004;82: 1022–8.
[31] Butler WR, Katz LS, Arriola J, Milvae RA, Foote RH. On the negative feedback regulation of gonadotropins in castrate and intact cattle with comparison of two FSH radioimmunoassays. J Anim Sci 1983;56:919–29.
[32] Wolfe MW, Roberson MS, Stumpf TT, Kittok RJ, Kinder JE. Circulating concentrations and pattern of luteinizing hormone and follicle-stimulating hormone in circulation are changed by the circulating concentration of 17 beta-estradiol in the bovine male and female. J Anim Sci 1992;70:248–53.
[33] Barnes MA, Kazmer GW, Bierley ST. Gonadotropic and ovarian hormone response in dairy cows treated with norgestomet and estradiol valerate. Theriogenology 1981;16:13–25.
[34] Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J Reprod Fertil 1992;94:177–88.
[35] Chaubal SA, Molina JA, Ohlrichs CL, Ferre LB, Faber DC, Bols PE, et al. Comparison of different transvaginal ovum pick-up protocols to optimise oocyte retrieval and embryo production over a 10-week period in cows. Theriogenology 2006;65: 1631–48.
[36] St. Clair EN, Patterson DJ, Schillo KK. Progestogen treatment stimulates follicle growth without affecting LH secretion in prepubertal beef heifers. J Anim Sci 1995;73(Suppl 1):222.

[37] Rahe CH, Owens RE, Fleeger JL, Newton HJ, Harms PG. Pattern of plasma luteinizing hormone in the cyclic cow: dependence upon the period of the cycle. Endocrinology 1980;107: 498–503.

[38] Garcia-Winder M, Lewis PE, Deaver DR, Smith VG, Lewis GS, Inskeep EK. Endocrine profiles associated with life span of induced corpora lutea in postpartum beef cows. J Anim Sci 1986;62:1353–62.

[39] Pfeifer LF, Mapletoft RJ, Adams GP, Kastelic JP, Small JA, Dias F, Singh J. The effect of the progesterone on follicular development and pregnancy rate in beef cattle. Reprod Fertil Dev 2008;20:90.

[40] Kojima FN, Chenault JR, Wehrman ME, Bergfeld EG, Cupp AS, Werth LA, et al. Melengestrol acetate at greater doses than typically used for estrous synchrony in bovine females does not mimic endogenous progesterone in regulation of secretion of luteinizing hormone and 17 beta-estradiol. Biol Reprod 1995;52:455–63.

[41] Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J Dairy Sci 2004;87:905–20.

[42] Gonzalez-Padilla E, Wiltbank JN, Niswender GD. Puberty in beef heifers. The interrelationship between pituitary, hypothalamic and ovarian hormones. J Anim Sci 1975;40:1091–104.

[43] Berardinelli JG, Dailey RA, Butcher RL, Inskeep EK. Source of progesterone prior to puberty in beef heifers. J Anim Sci 1979;49:1276–80.

[44] Kastelic JP, Colazo MG, Martinez MF, Mapletoft RJ. Effects of a CIDR or MGA, reproductive status, and estradiol, GnRH or pLH treatment on pregnancy rate to fixed-time AI in beef heifers. Theriogenology 2003;59:223.

ELSEVIER

Animal Reproduction Science 109 (2008) 100–109

ANIMAL REPRODUCTION SCIENCE

www.elsevier.com/locate/anireprosci

Superovulatory response in a bovine model of reproductive aging

Pritpal S. Malhi [a], Gregg P. Adams [a], Reuben J. Mapletoft [b], Jaswant Singh [a,*]

[a] *Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada S7N 5B4*

[b] *Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada S7N 5B4*

Received 25 October 2006; received in revised form 3 December 2007; accepted 13 December 2007
Available online 23 December 2007

Abstract

Two experiments were done to test the hypotheses that aging in cattle is associated with a reduced number of follicles recruited into an ovarian follicular wave, and a reduction in the ovarian response to gonadotropin treatment. Older cows (13–16 years of age) and their daughters (3–6 years of age) were treated with FSH for ovarian superstimulation four times over two consecutive years (31 and 33 superstimulations in old and young cows, respectively, experiments and years combined). In Experiment 1, ovulation was induced using LH. In Experiment 2, cumulus-oocyte complexes were collected by ultrasonographic-guided follicle aspirations before expected ovulations. The ovarian follicular and ovulatory responses were monitored daily by ultrasonography. Fewer 2–5 mm follicles ($P < 0.01$) were detected at the expected time of follicular wave emergence in older cows than in their daughters. After superstimulation, older cows had fewer follicles $\geq$6 mm ($P < 0.01$), and tended ($P = 0.1$) to have fewer ovulations than their daughters (32 ± 4 versus 40 ± 3, respectively). There was a positive correlation in the response of individual cows to successive superstimulatory treatments ($r > 0.8$; $P < 0.0001$) and the number of detected ovulations from one year to the next ($r = 0.6$; $P = 0.04$). In conclusion, aging was associated with fewer 2–5 mm follicles at follicular wave emergence and a lesser follicular and ovulatory response after superstimulatory treatment. The follicular and ovulatory response after superstimulation was repeatable within individuals, regardless of age.
© 2008 Elsevier B.V. All rights reserved.

Keywords: Aging; Bovine model; Ovary; Superstimulation; Superovulation

* Corresponding author. Tel.: +1 306 966 7410; fax: +1 306 966 7405.
E-mail address: jaswant.singh@usask.ca (J. Singh).

0378-4320/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.anireprosci.2007.12.002

1. Introduction

Development of ovarian follicular waves and methods to control wave emergence have been well characterized in cattle (Bo et al., 1995; Adams, 1999; Ginther et al., 2001; Mapletoft et al., 2002). In the majority of bovine interovulatory intervals, either two or three waves of follicular development have been reported (Ginther et al., 1989; Singh and Adams, 1998). Critically derived information on the effects of reproductive aging on follicular dynamics in cattle is sparse, but in a recent study (Malhi et al., 2005), circulating concentrations of FSH were greater and the number of small follicles recruited into each wave was less in older cows compared to their daughters. However, the follicular wave pattern was maintained in 13- and 14-year-old cattle (Malhi et al., 2005).

The goal of ovarian superstimulation is to obtain multiple, developmentally competent oocytes for *in vivo* or *in vitro* embryo production. Using ultrasonography, the number of antral follicles $\geq$2 mm at the start of superstimulatory treatment was highly predictable of the superstimulatory response in cattle (Cushman et al., 1999; Singh et al., 2004; De Roover et al., 2005) and women (Ng et al., 2000).

The mechanisms of follicular wave emergence, selection of a dominant follicle, and ovulation were fundamentally similar in cattle and women (Adams and Pierson, 1995; Baerwald et al., 2003a). The majority of menstrual cycles in women were also composed of two or three follicular waves (Baerwald et al., 2003a,b). In all follicular waves of natural estrous cycles in cattle, and in major waves during the menstrual cycle in women, one follicle is selected to become dominant while the others (subordinates) regress (Adams et al., 1993; Baerwald et al., 2003a). In addition, the earliest sign of reproductive aging in women (Klein et al., 1996) was an increase in circulating concentrations of FSH.

The circulating concentrations of anti-Mullerian hormone may be a more precise indicator of the reproductive age, and of the ovarian primordial follicle reserve, in animals as well as in humans (Kevenaar et al., 2006; La Marca and Volpe, 2006). A decline in the antral follicle count (Scheffer et al., 1999), a reduced ovarian superstimulatory response, and lesser subsequent ova/embryo recovery has been observed in older women (Kim, 1995; Dew et al., 1998). Similarly, older cows tended to have fewer $\geq$9 mm follicles after ovarian superstimulation (Malhi et al., 2006). Based on these similarities, a bovine model was proposed to study ovarian function and age-associated subfertility in women (Adams and Pierson, 1995; Malhi et al., 2005). In addition, the data from this study will be helpful to researchers and bovine practitioners to consider maternal aging while planning superstimulatory treatments.

The present study was designed to test the hypotheses that aging in cattle is associated with (1) a reduction in the number of small antral follicle (2–5 mm) recruited into an ovarian follicular wave, and (2) a reduced follicular and ovulatory response following gonadotropin treatment. In a previous study, approximately 55% of the cows were classified as infertile by 13 years of age (Erickson et al., 1976). However, older cows used in the present study were selected for fertility; i.e., other cows in the herd that had failed to produce a calf annually were culled. Thus, the group of 13- to 15-year-old cows in the present study represents the top half of the herd in terms of fertility. Age-dependent reproductive traits can be affected by within- and between-individual variations (van de Pol and Verhulst, 2006) therefore, to minimize the confounding of inherent fertility in this group of cows, comparisons were made using their daughters.

2. Materials and methods

The experiments were conducted at the Goodale Research Farm, University of Saskatchewan, Saskatoon, SK, Canada (52° North and 106° West) during the months of May to July. The same groups of older cows and their daughters were treated to superstimulate ovarian follicular development four times in two consecutive years. The experiment was conducted in three to four replicates in both years. Mother–daughter pairs were superstimulated in the same replicate to distribute inter-replicate variation equally between age groups. Animals were at least 45 days post-partum, non-lactating, non-pregnant, and each had a corpus luteum at the beginning of the experimental period. All animals were maintained in a single outdoor corral. The experimental protocol was approved by the University Committee on Animal Care and Supply under guidelines of the Canadian Council on Animal Care.

2.1. Experiment 1

The experiment was conducted on crossbred Hereford cows (13–16 years old, $n = 6$ in Year 1 and $n = 9$ in Year 2) and their daughters (3–6 years old, $n = 8$ in Year 1 and $n = 9$ in Year 2). Mean (± S.E.M.) body weights in older cows and their daughters were 737 ± 16 kg and 775 ± 29 kg, respectively. Estradiol-17β and progesterone treatment was used to induce the emergence of a new follicular wave (Bo et al., 1994, 1995; Malhi et al., 2006). Briefly, estradiol-17β (5 mg; Catalog # E8875, Sigma Chemical Company, St. Louis, Missouri, USA) and progesterone (100 mg; Catalog # P0130, Sigma Chemical Company, St. Louis, Missouri, USA) were dissolved in benzyl alcohol (0.4 ml; Catalog # B27354, BDH Inc., Toronto, Ontario, Canada), mixed with canola oil (2 ml; No name®, Sunfresh Limited, Montreal, Quebec, Canada) and given intramuscularly. An intravaginal progesterone-releasing device containing 1.9 g progesterone (CIDR-B®, Pfizer Animal Health, Montreal, PQ, Canada) was inserted at the time of steroid treatment.

Ovarian superstimulation was induced using a standard 4-day FSH treatment protocol (Mapletoft et al., 2002) beginning at the expected time of follicle wave emergence; i.e., 4 days after estradiol 17-β and progesterone treatment (Bo et al., 1994; Mapletoft et al., 2002; Malhi et al., 2006). Cows were given a total dose of 50 mg NIH-FSH-P1 FSH/100 kg body weight as administered intramuscularly in the morning and evening over 4 days (i.e., eight treatments of 6.25 mg Folltropin-V®/100 kg body weight; Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada). The total FSH dose (mean ± S.E.M.) was 368 ± 8 mg in older cows, and 387 ± 14 mg in their daughters. On the last day of FSH treatment, 500 μg cloprostenol (Estrumate®, Schering-Plough Animal health, Pointe-Claire, Quebec, Canada) were given intramuscularly in the morning and evening, and CIDR were removed at the time of the second cloprostenol treatment. All cows were given 25 mg Armour standard LH (Lutropin-V®, Bioniche Animal Health Canada Inc.) 24 h after the second cloprostenol treatment to induce ovulation. All animals were artificially inseminated and embryos were collected for the purposes of a separate study.

To record follicular development, transrectal ovarian ultrasonographic examinations were performed every 24 h by the same operator using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900, Tokyo, Japan). Follicle numbers in the 2–5 mm, 6–8 mm, 9–11 mm, and ≥12 mm categories were counted during each examination. The number of ovulations was estimated by the disappearance of follicles ≥6 mm recorded during the previous ultrasound examination. In a previous study (Nasser et al., 1993), there was a significant correlation between numbers of ovulations detected using ultrasonography, and by counting corpora lutea at the time of slaughter. The cut-off of ≥6 mm diameter was chosen to minimize the possibility of

missing any ovulations, and in a previous study, the diameter of the dominant follicles in animals treated to superstimulate ovarian follicular development was smaller than single dominant follicle (Adams et al., 1993).

2.2. *Experiment 2*

The experiment was conducted after completion of Experiment 1 using the same group of older cows (13–16 years old, $n = 7$ in Year 1 and $n = 9$ in Year 2) and their daughters (3–6 years old, $n = 7$ in Year 1 and $n = 9$ in Year 2). Mean (± S.E.M.) body weights in older cows and their daughters were 738 ± 15 kg and 769 ± 30 kg, respectively. Sufficient time was allowed between experiments for each cow to complete one natural interovulatory interval. To induce ovulation thereafter, all cows in the luteal phase were given a single 500 μg dose of cloprostenol. The emergence of a new follicular wave was induced 5–7 days after ovulation by transvaginal ultrasonographic-guided ablation of all follicles ≥5 mm (Bergfelt et al., 1997). Superstimulatory treatment with FSH was initiated at the expected time of wave emergence; i.e., 24 h after follicular ablation (Bergfelt et al., 1997; Mapletoft et al., 2002). Cows were given a total dose of 44 mg NIH-FSH-P1 FSH/100 kg body weight as administered intramuscularly in the morning and evening for 3.5 days (i.e., seven treatments of 6.25 mg Folltropin-V®/100 kg body weight). The total FSH dose (mean ± S.E.M.) was 323 ± 7 mg in old cows, and 337 ± 13 mg in daughters. Follicular development was recorded by transrectal ovarian ultrasonographic examinations as described in Experiment 1. For the purposes of a separate study, cumulus-oocyte complexes were aspirated from follicles ≥5 mm by transvaginal ultrasound-guided needle puncture 48–72 h after the last FSH treatment. Thus, superovulation data were not available from Experiment 2.

2.3. *Data analysis*

Follicle number data from superstimulation of ovarian follicular development (Experiments 1 and 2) were combined to determine the effects of age, year, and day of treatment. Serial data on follicle numbers in different diameter categories were analyzed by analysis of variance for repeated measures (Littell et al., 2000; Malhi et al., 2006; Littell, 2006) using the mixed procedure of the Statistical Analysis System (SAS version 8.2 for MS Windows, SAS Institute Inc., Cary, North Carolina, USA). The repeated observations from the same animals were used as independent records as (1) observational periods were separated in time by 1 month or 1 year, and (2) although the cows were the same, the follicles that responded to FSH treatments were independent in successive treatment periods. Moreover, the animal model used is rare; that is, older and younger mother–daughter pairs are not readily available. Hence, it was not possible to use a separate group for each superstimulatory period. An additional analysis for repeated measures (i.e., repeated follicle counts in four successive superstimulatory periods only from the Day 5 of superstimulation) was performed to determine if the interpretation of results were valid.

Single point numerical data (e.g., follicle numbers, ovulations) were compared between older cows and their daughters by paired *t*-tests, Student's *t*-tests, and the general linear model of SAS. Proportional data on ovulations were compared by chi-square analysis. Pearson's correlation for the number of follicles ≥6 mm (observed 5 days after the start of FSH treatment) between Experiments 1 and 2 and between Years 1 and 2 was performed using the correlation procedure in SAS. Similarly, the correlation between the numbers of ovulations in 2 years of Experiment 1 was calculated. Values are expressed as the mean ± S.E.M. unless otherwise specified.

3. Results

The mean body weight and the total FSH dose given to older cows and to their daughters in Experiments 1 and 2 were not different ($P > 0.3$). Fewer 2–5 mm follicles were detected at the time of initiation of FSH treatment (Day 0) in older cows than in their daughters (23 ± 2 versus 28 ± 2, $n = 29$ mother–daughter pairs; $P = 0.006$). Fewer follicles were observed in older cows than in their daughters in the 6–8 mm, 9–11 mm, and ≥12 mm categories ($P < 0.05$; Fig. 1B–D). The lesser number of 9–11 mm and ≥12 mm follicles was attributed to differences on Days 4 and 5 (age × day interaction, $P \leq 0.05$; Fig. 1C and D). Furthermore, fewer follicles ≥6 mm were observed on Day

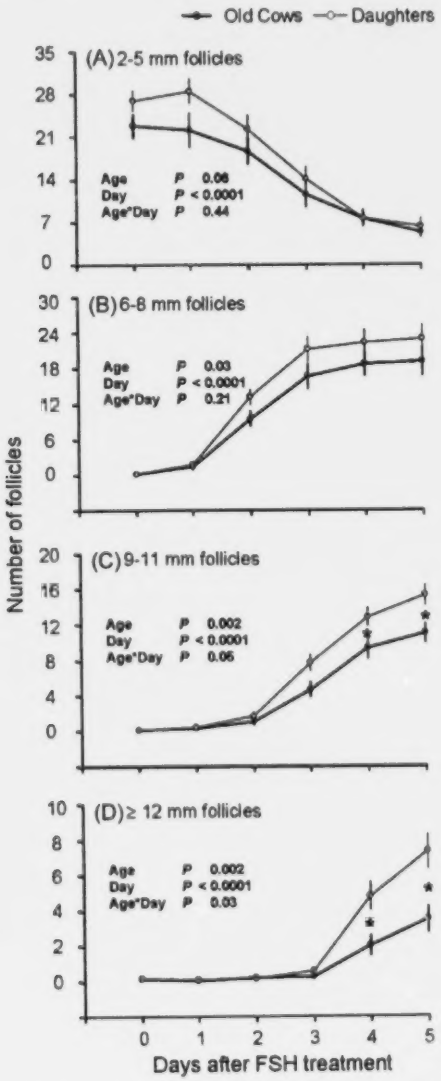


Fig. 1. Number (mean ± S.E.M.) of 2–5 mm follicles (A), 6–8 mm follicles (B), 9–11 mm follicles (C), and ≥12 mm follicles (D) in older cows ($n = 31$ superstimulatory cycles) and their daughters ($n = 33$ superstimulatory cycles) undergoing ovarian superstimulation. Data were analyzed relative to the start of FSH treatment (Day 0). *Values between groups differ ($P \leq 0.05$).

5 in older cows than in their daughters (34 ± 3 versus 46 ± 3, $n = 29$ mother–daughter pairs; $P = 0.0004$). These results were validated after a different repeated measures analysis where follicle count data (i.e., only Day 5) from four successive ovarian superstimulations was compared between older cows and their daughters. Numbers of ≥6 mm, 9–11 mm, and ≥12 mm follicles on Day 5 of superstimulation were less ($P < 0.01$) in older cows than their daughters.

When follicle number data from both experiments were compared between Years 1 and 2, more 2–5 mm follicles were detected in Year 2 than in Year 1 ($P = 0.01$; Fig. 2A). Similarly, more 6–8 mm follicles were observed on Days 4 and 5 of observational period in Year 2 than in Year 1 ($P < 0.05$, specific day comparison; Fig. 2B). Conversely, fewer 9–11 mm and ≥12 mm follicles

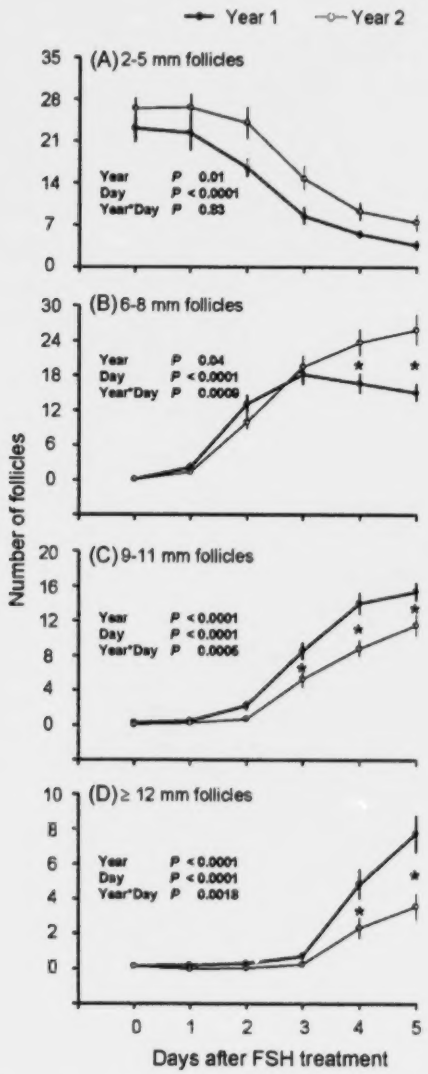


Fig. 2. Number (mean ± S.E.M.) of 2–5 mm follicles (A), 6–8 mm follicles (B), 9–11 mm follicles (C), and ≥12 mm follicles (D) in Year 1 ($n = 28$ superstimulatory cycles) and Year 2 ($n = 36$ superstimulatory cycles) undergoing ovarian superstimulation. Data were analyzed relative to the start of FSH treatment (Day 0). *Values between groups differ ($P \leq 0.05$).

Table 1
Data (mean ± S.E.M.) for superstimulatory treatment of older cows and their daughters ($n = 14$, mother–daughter pairs; Experiment 1)

	Older cows	Daughters	*P*-value
Number of:			
Follicles ≥6 mm 5 days after start of FSH treatment	39 ± 4	54 ± 4	<0.01
Ovulations*	32 ± 4	40 ± 3	0.11
Proportion of:			
Ovulations from follicles ≥6 mm	0.81	0.74	0.24
Ovulations detected within 24 h of pLH treatment	0.03	0.02	0.10
Ovulations detected between 24 h and 48 h after pLH	0.84	0.94	0.04
Ovulations detected between 48 h and 72 h after pLH	0.13	0.04	0.04

*Ovulation was detected by ultrasonography; i.e., by the disappearance of follicles ≥6 mm recorded during the previous ultrasound examination.

were observed in Year 2 than in Year 1 ($P < 0.05$, specific day comparison; Fig. 2C and D). When numbers of 6–8 mm, 9–11 mm, and ≥12 mm follicles on Day 5 were analyzed for age and year effect, there was no differential effect of year on follicle numbers in old cows and their daughters (age × year interaction, $P > 0.5$).

Data from Experiment 1 were also analyzed separately to study follicle numbers in relation to ovulations (Table 1). Most of the ovulations (84–94%) in older cows and their daughters were detected between 24 h and 48 h after LH treatment. The difference in the proportion of ovulations detected at different intervals (Table 1) was attributed primarily to one older cow in which most of the ovulations (Year 1 = 83%, Year 2 = 91%) were detected between 48 h and 72 h after LH treatment. When data from this cow were excluded, the percentage of ovulations between 48 h and 72 h after LH treatment did not differ between age groups (4% compared with 4%).

Irrespective of age, the responses of individual cows (measured by number of ≥6 mm follicles detected on Day 5) after successive ovarian superstimulations were positively correlated between Years 1 and 2 ($r = 0.8$; $P < 0.0001$), and between Experiments 1 and 2 ($r = 0.9$; $P < 0.0001$). Similarly, the number of ovulations within animal in Years 1 and 2 of Experiment 1 were also positively correlated ($r = 0.6$; $P = 0.04$).

4. Discussion

Over a decade ago, the bovine model was proposed for the study of ovarian function in women (Adams and Pierson, 1995), and the model formed the basis of recent discoveries of follicular wave dynamics in women (Baerwald et al., 2003a,b). Follicular and endocrine events of the normal reproductive cycle (i.e., follicular wave emergence, selection, and ovulation) were fundamentally similar between cattle and women (Adams et al., 1992; Adams and Pierson, 1995; Baerwald et al., 2003a,b). In women, circulating concentrations of FSH (Klein et al., 1996) and anti-Mullerian hormone (La Marca and Volpe, 2006) are considered as indicators of reproductive aging, and of the ovarian primordial follicle reserve. Likewise, an increase in circulating FSH was detected in older cows (13–14 years) with no visible signs of infertility (Malhi et al., 2005). However, circulating concentrations of anti-Mullerian hormone were not evaluated in the previous study (Malhi et al., 2005). A reduction in the number of 4 mm and 5 mm follicles at the time of follicle wave emergence was also observed in the same group of aged cattle.

The present experiments were designed to study the effect of reproductive aging on recruitment of small (2–5 mm) antral follicles into follicular waves, and to compare follicular and ovulatory response in older cows and their daughters. The mother–daughter pairs were used to reduce the effects of genetic variation. In support of the stated hypothesis, fewer 2–5 mm follicles were detected in older cows than in their daughters at the expected time of follicular wave emergence. Consistent with a lesser count of 2–5 mm follicles at wave emergence in the present study, significantly fewer 6–8 mm, 9–11 mm, and ≥12 mm follicles were observed during the treatment period in older cows than in their daughters. Results were also consistent with other studies in which the antral follicle count (all follicles ≥2 mm diameter) was highly predictive of the ovarian superstimulatory response in cattle (Singh et al., 2004; Burns et al., 2005) and in women (Ng et al., 2000). Women with a lesser follicular response to ovarian superstimulation have been reported to show more signs of reproductive aging (Beckers et al., 2002), and enter into menopause earlier than those with a greater follicular response within the same age groups (de Boer et al., 2002).

When combined over both experiments, fewer ≥6 mm follicles were detected at the end of superstimulatory treatment in older cows than in their daughters. A similar effect was observed in a separate analysis of data from Experiment 1. The fewer number of follicles in older cows compared with their daughters was consistent with the tendency for a lesser ovulation rate in older cows; on average, younger cows had eight more ovulations per cow than older cows. Thus, the hypothesis that reproductive aging is associated with a reduced follicular and ovulatory response after gonadotropin treatment was supported.

The proportion of ≥6 mm follicles that ovulated was not different when older cows and their daughters were compared, suggesting equal responsiveness to LH between age groups. There was a highly positive correlation in the response of individual cows to successive ovarian superstimulatory treatments, and the number of detected ovulations over years. Results are consistent with previous studies in cattle where the number of 2–6 mm follicles at follicular wave emergence was significantly correlated with their number in successive waves (Singh et al., 2004), and the repeatability index for the number of CL counted and the number of viable embryos recovered after ovarian superstimulation was over 0.39 and 0.30, respectively (Benyei et al., 2004; Peixoto et al., 2004). The repeatability for superovulatory response was also greater in sheep (Bari et al., 2001), but is in contrast to a study in Holstein cattle (Tonhati et al., 1999) in which the repeatability of the superovulatory response, based on numbers of transferable embryos, was low (0.13).

In summary, reproductive aging in cattle was associated with a reduced number of small antral follicles at the time of follicular wave emergence, and a reduced superstimulatory and superovulatory response. The proportion of large follicles from which ovulation occurred was identical between age groups suggesting no age-related deterioration in the ovulatory mechanism. The previous superstimulatory and superovulatory response of individual cows was predictive of subsequent responses. Based on consistent age-related effects on ovarian function, the bovine model may be as useful for the study of reproductive senescence in humans as it has been for elucidating follicle dynamics during the natural menstrual cycle. In particular, the bovine model will aid in future studies of oocyte-associated subfertility in aging women.

Acknowledgements

This study was supported by grants from the Saskatchewan Agriculture Development Fund, Saskatchewan Health Research Foundation, and the Natural Sciences and Engineering Research Council of Canada. We gratefully acknowledge Bioniche Animal Health Canada Inc., Belleville, Ontario, Canada for providing hormones for the study, and Bill Kerr and his staff at the Goodale

Research Farm, University of Saskatchewan for animal care and maintenance. We also thank Durairaj Saravanan, Andrew McCarthy, Nicole Wensink, Melissa Maximnuk, Ross Hawkes, Laura Marshall, and Jody Barry for their assistance with data collection.

References

Adams, G.P., 1999. Comparative patterns of follicle development and selection in ruminants. J. Reprod. Fertil. Suppl. 54, 17–32.

Adams, G.P., Kot, K., Smith, C.A., Ginther, O.J., 1993. Selection of dominant follicle and suppression of follicular growth in heifers. Anim. Reprod. Sci. 30, 259–271.

Adams, G.P., Matteri, R.L., Kastelic, J.P., Ko, J.C., Ginther, O.J., 1992. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. J. Reprod. Fertil. 94, 177–188.

Adams, G.P., Pierson, R.A., 1995. Bovine model for study of ovarian follicular dynamics in humans. Theriogenology 43, 113–120.

Baerwald, A.R., Adams, G.P., Pierson, R.A., 2003a. Characterization of ovarian follicular wave dynamics in women. Biol. Reprod. 69, 1023–1031.

Baerwald, A.R., Adams, G.P., Pierson, R.A., 2003b. A new model for ovarian follicular development during the human menstrual cycle. Fertil. Steril. 80, 116–122.

Bari, F., Khalid, M., Wolf, B., Haresign, W., Murray, A., Merrel, B., 2001. The repeatability of superovulatory response and embryo recovery in sheep. Theriogenology 56, 147–155.

Beckers, N.G., Macklon, N.S., Eijkemans, M.J., Fauser, B.C., 2002. Women with regular menstrual cycles and a poor response to ovarian hyperstimulation for in vitro fertilization exhibit follicular phase characteristics suggestive of ovarian aging. Fertil. Steril. 78, 291–297.

Benyei, B., Gaspardy, A., Komlosi, I., Pecsi, A., 2004. Repeatability and heritability of ovulation number and embryos in dam-daughters pairs in superovulated Holstein–Friesian cows. Reprod. Domest. Anim. 39, 99–102.

Bergfelt, D.R., Bo, G.A., Mapletoft, R.J., Adams, G.P., 1997. Superovulatory response following ablation-induced follicular wave emergence at random stages of the oestrous cycle in cattle. Anim. Reprod. Sci. 49, 1–12.

Bo, G.A., Adams, G.P., Pierson, R.A., Mapletoft, R.J., 1995. Exogenous control of follicular wave emergence in cattle. Theriogenology 43, 32–40.

Bo, G.A., Adams, G.P., Pierson, R.A., Tribulo, H.E., Caccia, M., Mapletoft, R.J., 1994. Follicular wave dynamics after estradiol-17ß treatment of heifers with or without progestogen implant. Theriogenology 41, 1555–1569.

Burns, D.S., Jimenez-Krassel, F., Ireland, J.L., Knight, P.G., Ireland, J.J., 2005. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. Biol. Reprod. 73, 54–62.

Cushman, R.A., DeSouza, J.C., Hedgpeth, V.S., Britt, J.H., 1999. Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the cow. Biol. Reprod. 60, 349–354.

de Boer, E.J., den Tonkelaar, I., te Velde, E.R., Burger, C.W., Klip, H., van Leeuwen, F.E., 2002. A low number of retrieved oocytes at in vitro fertilization treatment is predictive of early menopause. Fertil. Steril. 77, 978–985.

De Roover, R., Bols, P.E., Genicot, G., Hanzen, C., 2005. Characterisation of low, medium and high responders following FSH stimulation prior to ultrasound-guided transvaginal oocyte retrieval in cows. Theriogenology 63, 1902–1913.

Dew, J.E., Don, R.A., Hughes, G.J., Johnson, T.C., Steigrad, S.J., 1998. The influence of advanced age on the outcome of assisted reproduction. J. Assist. Reprod. Genet. 15, 210–214.

Erickson, B.H., Reynolds, R.A., Murphree, R.L., 1976. Ovarian characteristics and reproductive performance of the aged cow. Biol. Reprod. 15, 555–560.

Ginther, O.J., Beg, M.A., Bergfelt, D.R., Donadeu, F.X., Kot, K., 2001. Follicle selection in monovular species. Biol. Reprod. 65, 638–647.

Ginther, O.J., Kastelic, J.P., Knopf, L., 1989. Composition and characteristic of follicular waves during the bovine estrus cycle. Anim. Reprod. Sci. 20, 187–200.

Kevenaar, M.E., Meerasahib, M.F., Kramer, P., van de Lang-Born, B.M., de Jong, F.H., Groome, N.P., Themmen, A.P., Visser, J.A., 2006. Serum anti-Mullerian hormone levels reflect the size of the primordial follicle pool in mice. Endocrinology 147, 3228–3234.

Kim, S.H., 1995. Female aging and superovulation induction for IVF. J. Obstet. Gynaecol. 21, 75–82.

Klein, N.A., Battaglia, D.E., Fujimoto, V.Y., Davis, G.S., Bremner, W.J., Soules, M.R., 1996. Reproductive aging: accelerated ovarian follicular development associated with a monotropic follicle-stimulating hormone rise in normal older women. J. Clin. Endocrinol. Metab. 81, 1038–1045.

La Marca, A., Volpe, A., 2006. Anti-Mullerian hormone (AMH) in female reproduction: is measurement of circulating AMH a useful tool? Clin. Endocrinol. (Oxf.) 64, 603–610.

Littell, R.C., 2006. SAS for Mixed Models, second ed. SAS Institute Inc., Cary, N.C.

Littell, R.C., Pendergast, J., Natarajan, R., 2000. Modelling covariance structure in the analysis of repeated measures data. Stat. Med. 19, 1793–1819.

Malhi, P.S., Adams, G.P., Pierson, R.A., Singh, J., 2006. Bovine model of reproductive aging: response to ovarian synchronization and superstimulation. Theriogenology 66, 1257–1266.

Malhi, P.S., Adams, G.P., Singh, J., 2005. Bovine model for the study of reproductive aging in women: follicular, luteal, and endocrine characteristics. Biol. Reprod. 73, 45–53.

Mapletoft, R.J., Steward, K.B., Adams, G.P., 2002. Recent advances in the superovulation in cattle. Reprod. Nutr. Dev. 42, 601–611.

Nasser, L., Adams, G.P., Bo, G.A., Mapletoft, R.J., 1993. Ovarian superstimulatory response relative to follicular wave emergence in heifers. Theriogenology 40, 713–724.

Ng, E.H., Tang, O.S., Ho, P.C., 2000. The significance of the number of antral follicles prior to stimulation in predicting ovarian responses in an IVF programme. Hum. Reprod. 15, 1937–1942.

Peixoto, M.G., Pereira, C.S., Bergmann, J.A., Penna, V.M., Fonseca, C.G., 2004. Genetic parameters of multiple ovulation traits in Nellore females. Theriogenology 62, 1459–1464.

Scheffer, G.J., Broekmans, F.J., Dorland, M., Habbema, J.D., Looman, C.W., te Velde, E.R., 1999. Antral follicle counts by transvaginal ultrasonography are related to age in women with proven natural fertility. Fertil. Steril. 72, 845–851.

Singh, J., Adams, G.P., 1998. Immunohistochemical distribution of follistatin in dominant and subordinate follicles and the corpus luteum of cattle. Biol. Reprod. 59, 561–570.

Singh, J., Dominguez, M., Jaiswal, R., Adams, G.P., 2004. A simple ultrasound test to predict the superstimulatory response in cattle. Theriogenology 62, 227–243.

Tonhati, H., Lobo, R.B., Oliveira, H.N., 1999. Repeatability and heritability of response to superovulation in Holstein cows. Theriogenology 51, 1151–1156.

van de Pol, M., Verhulst, S., 2006. Age-dependent traits: a new statistical model to separate within- and between-individual effects. Am. Nat. 167, 766–773.

Available online at www.sciencedirect.com

ScienceDirect

Theriogenology

Theriogenology 69 (2008) 72–80

www.theriojournal.com

Progress in understanding ovarian follicular dynamics in cattle

G.P. Adams [a,*], R. Jaiswal [b], J. Singh [a], P. Malhi [c]

[a] *Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada*
[b] *Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada*
[c] *Canadian Food Inspection Agency, Lethbridge, Alberta, Canada*

Abstract

The study of follicular dynamics began in the mid-20th century, but progress has been particularly rapid in the last two decades through the use of tools that have enabled serial, non-invasive examination. A brief overview of early oogenesis and folliculogenesis is provided as a backdrop to the evolution of our understanding of follicular dynamics during the bovine estrous cycle. Studies to date support the concept that the pair of ovaries acts as a single unit and influences follicular development primarily via systemic endocrine routes involving ovarian and uterine products, the gonadotropins, and their receptors. Dominant and subordinate follicles pass through growing, static and regressing phases that have distinct morphologic and biochemical characteristics; these changes are the basis of efforts focused on diagnosing and manipulating follicular status. An update of research progress highlights recent findings on the repeatability (predictability) within individuals of follicle recruitment and wave pattern (two- versus three-wave cycles), the relationship between oocyte competence and follicular status, and the dynamics of small follicles. Recent studies documented that wave emergence and follicular dominance are apparent earlier than previously reported, and on the basis of periodic endogenous FSH surges and the presence of FSH receptors, the hypothesis that follicles become progressively entrained to waves from the earliest stages of development is introduced. Lastly, recent studies comparing old cows and their young daughters provide a new understanding of the effects of aging on gonadotropins and ovarian steroids, follicular dynamics, ovarian response to synchronization, superstimulation, and oocyte competence.
© 2007 Elsevier Inc. All rights reserved.

Keywords: Follicle dynamics; Estrous cycle; Bovine; Oocyte competence; Reproductive aging

1. Introduction—a history of ovarian research

Controversy has been a hallmark of the study of ovarian form and function from the earliest descriptions of the female gonad [1]. In the 5th century B.C., Hippocrates did not ascribe any generative role of the ovary, but rather suggested that generation of a new life was the result of the action of two kinds of semen—one from the male (ejaculate) and one from the female (menstrual blood). A century later, Aristotle characterized the ovary as an imperfect vestige of the male testis with no apparent function. It was not until the mid-1600s that the ovary was recognized for what it was—the producer of eggs. The Dutch physician, Regnier de Graaf, is often cited as the first to recognize the rightful role of the ovary in his "New treatise concerning the generative organs of women" published in 1672. The 31-year-old de Graaf, however, was embroiled in bitter arguments with his anatomy professor, Johannes van Horne, and classmate, Jan Swammerdam, who claimed that they had revealed the form (and by extension, the function) of the ovary in a short communication in 1668.

* Corresponding author at: Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 5B4. Tel.: +1 306 966 7411; fax: +1 306 966 7405.
E-mail address: gregg.adams@usask.ca (G.P. Adams).

0093-691X/$ – see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.theriogenology.2007.09.026

Their early scientific "abstract", however, was preceded by one of de Graaf's a few weeks earlier, in March 1668. Ironically, the conclusion of a committee tasked to settle the dispute was that neither de Graaf nor Van Horne was the first to have seen eggs in viviparous organisms, but rather another classmate, Niels Steno, who reported in his 1667 treatise that "the testicles of women are analogous to the ovary." All of these early modern scientists, however, held the mistaken belief that the follicle itself was the egg—like a small bird's egg without a shell. It was not until 1827 when an Estonian physician, Karl Ernst von Baer, provided the first description of a mammalian egg from his microscopic study of ovarian vesicles (follicles) in the ovary of a dog. The first studies of the dynamics of follicle development, however, were not for another 100 years.

The purpose of this report is to provide a brief overview and historical background of our understanding of follicle dynamics in cattle, and to highlight some recent advances, primarily from the author's laboratory. It is not intended to be a comprehensive review. For brevity, reference is made to reviews of specific topics rather than original studies. Unless otherwise stated, the information presented is from results of studies on *Bos taurus*.

2. Early oogenesis and folliculogenesis

Oocytes originate as primordial germ cells from the endoderm of the embryonic yolk sac, and migrate by amoeboid movement via the dorsal mesentery of the hindgut to the gonadal ridge [2] by Day 35 of gestation in cattle [3]. Primordial germ cells undergo a limited number of mitotic divisions during migration and upon arrival at the gonadal ridge [2,4]. Primordial germ cells are internalized into the gonadal ridge through its surface epithelium—initially thought to be the source of primordial germ cells and mistakenly named the "germinal" epithelium. During the process of internalization, the primordial germ cells cease mitotic division, become enclosed in germ cell cords (ovigerous cords) composed of epithelial cells which are delineated from the surrounding mesenchymal cells by a basal lamina, and they become referred to as oogonia [4]. Meiosis of oogonia (transition to primary oocytes) begins by Days 75–80 of gestation in cattle and the first meiotic division does not proceed beyond the pachytene stage of prophase-I [3], at which time the chromosomes are decondensed and contained within the nuclear membrane—the germinal vesicle [2].

A single layer of flattened epithelial cells from the germ cell cords condense around the vast majority of surviving oocytes and enclose them to form primordial follicles [3,4]. Oocytes that fail to be surrounded by epithelial cells degenerate [2]. Initiation of follicular growth (activation) begins with the transformation of the flattened pre-granulosa cells of the primordial follicle into a single layer of cuboidal granulosa (follicular) cells—a primary follicle [5]. Proliferation of granulosa cells results in an increase from two to six layers around the oocyte (secondary follicle), to >6 layers of granulosa cells and a fluid-filled antrum (tertiary or antral follicle) [5,6].

3. Follicular dynamics during the bovine estrous cycle

3.1. Follicular waves

The estrous cycle and its phases in cattle were first described by Hammond [7], followed by McNutt [8], and Cole [9]. In 1946, Bullough [10] used a mouse model and described the relationship between ovarian follicular development and hormones. Studies of the dynamics of follicular development were first reported in rats by Mandle and Zukerman [11] and in monkeys in 1951 by Green and Zukerman [12]. Both studies involved a histological approach and both concluded that there was no cyclic variation in follicle numbers. Rajakoski [13] has been credited with the initial proposition of the two-wave theory of follicular growth during the bovine estrous cycle. For three decades after Rajakoski's report, many experiments were done on various aspects of follicular dynamics during the bovine estrous cycle, resulting in contradicting accounts of the nature of follicle development, ranging from the absence of waves to as many as three or four distinct waves per estrous cycle. In later reviews, the two-wave theory of Rajakoski was refuted on the basis that "conclusions were based on qualitative assessment of data without current knowledge of the profile of gonadotropins and of ovarian steroids..." (reviewed in [14]). Evidence was presented to support the concept that follicles are recruited continuously throughout the cycle and the follicle destined to ovulate is selected by coincidence of its stage of maturity (readiness) and the occurrence of the preovulatory gonadotropin surge. However, with the introduction of ultrasonography in the late 1980s, the barrier to our understanding of follicular dynamics was suddenly broken (reviewed in [15]).

Studies using ultrasonic imaging to monitor follicle populations in different size categories or to monitor

individually identified follicles (reviewed in [14]) have convincingly documented that follicular growth in cattle occurs in a wave-like fashion and that the majority of estrous cycles in cattle are comprised of two or three such waves. Follicular wave emergence in cattle is characterized by the sudden (within 2–3 days) growth of 8–41 small follicles that are initially detected by ultrasonography at a diameter of 3–4 mm (Fig. 1) (reviewed in [14,16]). The growth rate is similar among follicles of the wave for approximately 2 days, when one follicle is selected to continue growth (dominant follicle), whereas the remainder become atretic and regress (subordinate follicles). Results of these early studies of follicle dynamics gave rise to the hypothesis that the dominant follicle suppresses the growth of the subordinates in the existing wave, and the emergence of the next follicular wave. Support for this hypothesis was provided in a series of studies involving systemic treatment with the proteinaceous fraction of follicular fluid and by electrocautery of the dominant follicle (reviewed in [17]). The applied implications of these findings were immediate and far-reaching, and marked a new era for ovarian synchronization and superstimulation in cattle [18,19].

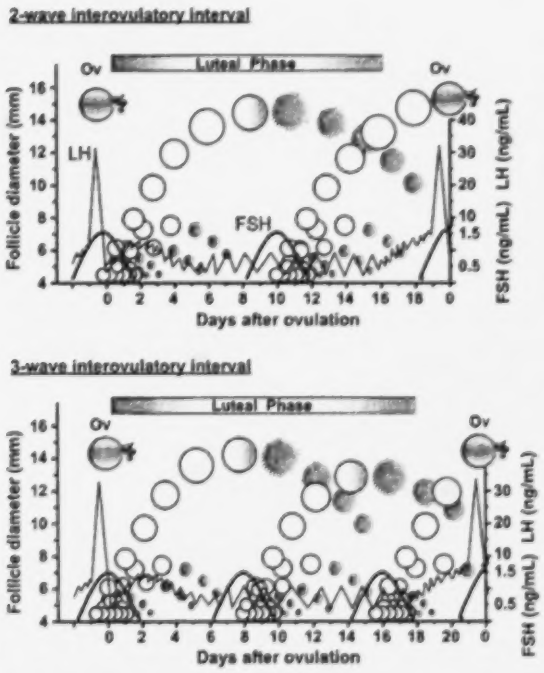


Fig. 1. Dynamics of ovarian follicular development and gonadotropin secretion during two- and three-wave estrous cycles in cattle. Dominant and subordinate follicles are indicated as open (viable) or shaded (atretic) circles. A surge in circulating FSH concentrations (thick line) precedes emergence of each wave. A surge in circulating LH concentrations (thin line) precedes ovulation. The LH surge is preceded and succeeded by a period of high-LH pulse frequency as a result of low-circulating progesterone concentrations (i.e., period of luteolysis and luteogenesis, respectively).

3.2. Two- versus three-wave patterns

The majority of bovine estrous cycles (i.e., >95%) are composed of either two or three follicular waves (reviewed in [19]). Some have reported a preponderance (>80%) of either the two- or three-wave pattern, whereas others have reported a more even distribution. In both two- and three-wave estrous cycles, emergence of the first follicular wave occurs consistently on the day of ovulation (Day 0). Emergence of the second wave occurs on Day 9 or 10 in two-wave cycles, and on Day 8 or 9 in three-wave cycles. In three-wave cycles, a third wave emerges on Day 15 or 16. Under the influence of progesterone (e.g., diestrus), dominant follicles of successive waves undergo atresia. The dominant follicle present at the onset of luteolysis becomes the ovulatory follicle, and emergence of the next wave is delayed until the day of the ensuing ovulation. The CL begins to regress earlier in two-wave cycles (Day 16) than in three-wave cycles (Day 19) resulting in a correspondingly shorter estrous cycle (19–20 days versus 22–23 days). Hence, the so-called 21-day-estrous cycle of cattle exists only as an average between two- and three-wave cycles (Fig. 1).

Predictive factors associated with a two- versus three-wave pattern may provide insight into mechanisms controlling the pattern, and have important implications on breeding management and the development of effective protocols for ovarian synchronization. Pregnancy rates in cattle with two- versus three-wave patterns were compared based on the notion that the preovulatory follicle in the two-wave pattern grows for a relatively longer period and may contain a relatively aged oocyte. However, results have been contradictory; pregnancy rates did not differ between two- versus three-wave cycles in some studies [20,21], whereas a lower pregnancy rate was reported for two-wave cycles in another study [22]. There appears to be no breed- or age-specific predilection for a given wave pattern in *B. taurus* cattle. An increase in the proportion of three-wave patterns has been associated with a low plane of nutrition and heat stress (reviewed in [19,23]). In *Bos indicus* cattle, no seasonal effect on wave pattern was detected, but the pattern was influenced by parity (reviewed in [23]). The majority of Nelore heifers (65%) exhibited a three-wave pattern, whereas the majority of Nelore cows (83%) exhibited a two-wave pattern. Others have

reported that up to 27% of estrous cycles in *B. indicus* cows consisted of four waves of follicular development, compared to 7% in *B. indicus* heifers (reviewed in [23]).

In a recent study involving ultrasonographic data from 91 interovulatory intervals [24], two- and three-wave patterns of follicular development were compared to determine the repeatability and predictive characteristics of a given wave pattern. Two-wave cycles were nearly 3 days shorter than three-wave cycles (19.8 ± 0.2 versus 22.5 ± 0.3; $P < 0.01$). The majority of cycles $\leq$21 days (88%) were of the two-wave pattern ($P < 0.05$), whereas the majority of cycles $\geq$22 days (78%) were of the three-wave pattern ($P < 0.05$). The proportion of serial cycles in which the pattern remained the same (i.e., repeatability) was more than twofold greater than the proportion cycles that changed patterns (70% versus 30%; $P < 0.01$). The repeatability of wave pattern, and the proportion of two- versus three-wave patterns within the herd were not affected by the season of year. The strongest correlate to the number of waves in an interovulatory interval was the duration of follicular dominance of Wave 1. The duration of dominance (defined as the period of the growing and static phases of the dominant follicle) was 3 days longer and the onset of regression was later in two-wave patterns than in three-wave patterns ($P < 0.01$). Dominance of Wave 1 was associated with a subsequent delay in the attainment of maximum diameter by the dominant follicle of Wave 2, as well as early onset of luteolysis. Therefore, factors that influence the development of the dominant follicle of Wave 1 may be responsible for regulating the wave pattern.

4. Hormonal interplay controlling wave dynamics, ovulation and CL development

4.1. Local versus systemic control

The two ovaries act primarily as a single unit; i.e., each follicular wave includes follicles from both ovaries that respond in unison. In a critical study of intra-ovarian relationships [25], the authors concluded that the dominant follicle suppressed subordinates and new wave emergence via systemic (endocrine) rather than local channels. Only one follicle from the pair of ovaries is selected to become dominant, the side of dominant follicle development was random, and the dominant follicle was equally likely to reside in the same or contralateral ovary to that of the largest subordinate follicle. The side of the CL or dominant follicle of a previous wave had no effect on the side of the ovulatory follicle. Although intrafollicular (autocrine and paracrine) factors are important for growth, health and demise of an individual follicle, there is no convincing in vivo documentation of one follicle affecting the health/regression status of its neighbors directly by a localized effect.

4.2. Gonadotropins and their receptors

Emergence of a follicular wave and selection of the dominant follicle are temporally associated with a rise and fall in circulating concentrations of FSH (Fig. 1) [17]. Emergence of a follicular wave is preceded by a surge in plasma FSH concentrations in both spontaneous waves and induced waves. Follicular products, especially those from the dominant follicle, are responsible for suppressing FSH release and, therefore, the emergence of the next follicular wave (Fig. 1). At the end of the period of dominance (i.e., at ovulation, or the mid-static phase of an anovulatory dominant follicle), circulating concentrations of FSH begin to rise; they increase 1.5–2-fold over the next 2 days, and peak approximately 12–24 h before emergence of the wave (when the future dominant follicle is 4–5 mm in diameter). If an existing dominant follicle is removed (i.e., follicular ablation), a surge in FSH begins within 12 h, resulting in emergence of a new follicular wave within next 24 h [26]. Selection of the dominant follicle is associated with decreasing blood FSH concentrations during the first 3 days of the wave. The nadir in FSH is reached 4 days after wave emergence, and concentrations remain low for the next 2–3 days. Receptors for FSH are present only on granulosa cells, whereas LH receptors are located on both granulosa and theca cells in the wall of antral follicles. The dominant follicle acquires more LH receptors on its granulosa cells than its subordinates and is therefore able to shift its gonadotropin dependence to LH during the FSH nadir, and continue to grow while the subordinates regress.

4.3. Ovarian and uterine products

The CL is the main source of progesterone; CL morphology and plasma progesterone concentration are good indicators of progesterone synthesis within the CL (reviewed in [15]). Intense angiogenesis, proliferation of granulosa and theca cells from the wall of ovulated follicle, and their differentiation (luteinization) during the first 5–6 days after ovulation (metestrus) results in a progressive increase in plasma progesterone concentration from <1 ng/mL at 3 days after ovulation to approximately 3 ng/mL by 6 days. Plasma progesterone concentration peaks between 10 and 14 days post-

ovulation (>4 ng/mL), followed by decreasing concentrations after 16 days due to luteolysis (hypoxic cell death resulting from hyalinization of blood vessels) induced by $PGF_{2\alpha}$ released from the endometrium of the nonpregnant cow.

Dominant follicles from both anovulatory and ovulatory waves produce estradiol. Theca cells are required for conversion of progesterone to androgens, whereas the aromatase enzyme (to convert androgen to estradiol) is exclusively localized in granulosa cells. After wave emergence, estradiol content in the follicular fluid of the growing dominant follicle increases at least 20-fold by the day of selection (3 days after wave emergence), followed by a threefold decrease by the early static phase of the anovulatory dominant follicle (6 days), before returning to base-line in the early regressing phase (11 days) [15]. Peak estradiol concentration in the follicular fluid of the ovulatory follicle is twice as high as the peak in anovulatory dominant follicles. Exogenous estradiol treatment during the luteal phase induces the demise of the existing dominant follicle, most likely by suppressing LH and FSH, followed by a rebound in FSH and synchronous emergence of a new follicular wave [14,18]. This treatment is the basis of many current protocols for ovulation synchronization for fixed-time AI and superstimulation. In addition to estradiol, which has a major inhibitory action on FSH, growing follicles produce other factors such as IGFs, inhibins and follistatin [27] that also regulate FSH release and availability. Although the dominant follicle plays a major role, all follicles of an emerging wave contribute to suppression of the wave-eliciting FSH surge [28].

The release of both FSH and LH is induced by pulses of GnRH from the hypothalamus, but because FSH release is profoundly influenced by follicular products and because its half-life in cattle is longer than that of LH, episodic release of FSH is less apparent than LH. Pulse frequency and amplitude of LH are influenced by circulating concentrations of both progesterone and estradiol. High levels of progesterone produced by a functional CL during diestrus or pregnancy suppress LH pulse frequency (Fig. 1). When plasma progesterone concentrations were approximately 1.7 ng/mL, six LH pulses per 8 h with an amplitude of 0.2 ng/mL were observed, whereas only 1.8 pulses per 8 h with an average amplitude of 0.34 ng/mL were observed when progesterone concentrations were 5 ng/mL [29]. Therefore, dominant follicles grow larger and remain dominant for a longer interval when LH pulse frequency is elevated (i.e., low progesterone) (reviewed in [14]). Increasing estradiol concentrations with decreasing progesterone after luteolysis increase the LH pulse frequency further, culminating in a large prevulatory LH surge.

5. Oocyte competence and follicular status

Morphological and cellular changes during follicular and luteal development are reflected in biochemical changes at the tissue and plasma levels, as well as in ultrasound image characteristics and the developmental competence of the oocyte (reviewed in [15]). Computer-assisted analyses of ultrasound images of dominant and subordinate follicles at different phases of development demonstrated that pixel heterogeneity of the antrum and wall, and the sharpness of the fluid–wall interface were negatively correlated ($P < 0.01$) with estradiol and the estradiol:progesterone ratio in follicular fluid [15]. Histologically, the wall thickness of Wave 1 dominant follicles decreased dramatically during the late-static (60 μm) and regressing (42 μm) phases, compared to the growing and early static phases (120 μm). Cells of the granulosa layer of the dominant follicle of Wave 1 became loose and degenerate during the late-static phase, and lined by fibroblast-like flattened cells during the regressing phase. The wall of subordinate follicles was thinner and had a lower mitotic index than that of the dominant follicle 3 and 6 days after wave emergence. The dominant follicle of Wave 1 displayed histological evidence of atresia at the time of emergence of Wave 2. Based on these data, follicular dominance is morphologically and biochemically maximal during the mid-growing phase (3 days after wave emergence), and begins to wane early in the static phase (6 days after wave emergence).

A positive relationship exists between early follicular atresia and oocyte competence [30,31]. A significantly greater proportion of cumulus–oocyte complexes collected from subordinate follicles during the early regressing phase were expanded, and a significantly greater proportion of oocytes showed evidence of nuclear maturation than those collected during the growing and early static phases. Furthermore, oocytes obtained from early regressing subordinate follicles (Day 5 of follicular wave) were more likely to develop into embryos in vitro than those obtained from growing (Day 2), early static (Day 3) or late-regressing (Day 7) subordinate follicles. Interestingly, ultrasound image gray-scale pixel values of the perifollicular stroma were lower in ovaries from which oocytes were collected that did not produce embryos compared to those that did produce embryos [31]. A comparison was made recently of the developmental

competence of oocytes that developed under low- versus high-progesterone concentrations during superstimulation with FSH [32]. Oocytes that developed under a high-progesterone milieu (i.e., low-LH pulse frequency) were less capable of developing into embryos. Interestingly, oocytes that developed under a prolonged-low-progesterone level (i.e., mimicking persistent oversized follicles) failed to ovulate. These findings have provided impetus for further studies to determine if short-term, low-progesterone exposure (i.e., high-LH pulse frequency) during the growing phase of follicle development after FSH superstimulation could be utilized to enhance the proportions of transferable embryos in vivo and in vitro.

6. Dynamics of small follicles

6.1. FSH responsiveness

Whereas the developmental dynamics of follicles ≥4 mm have been well characterized, the dynamics of smaller follicles remain a mystery. In a classic experiment involving assessment of the mitotic index of granulosa cells before and after colchicine treatment [6], it was estimated that small follicles grew more slowly (27 days to grow from 0.13 mm to 0.67 mm) than larger follicles (6.8 days to grow from 0.68 mm to 3.67 mm) and that two estrous cycles were required for preantral follicles to reach the mature antral stage. However, these estimations do not shed light on follicle dynamics in relation to changes in circulating concentrations of gonadotropins and follicular wave emergence.

Similarly, the role of FSH in regulating the wave-like development of follicles ≥4 mm has been clearly defined [17], whereas the role of FSH in smaller follicles, including small antral, and preantral follicles, remains unclear. An early study demonstrated the binding of FSH to the granulosa cells of follicles with only a single layer of granulosa cells [33], but it has been argued that they may not be coupled to the adenylate cyclase-second messenger system during early stages of folliculogenesis, and may be non-functional [34]. However, the growth-promoting effect of FSH on preantral and small antral follicles in cattle produced in vitro [35] and in vivo [36,37] suggests a role for FSH in the development of early stage preantral and antral follicles.

If small follicles are responsive to FSH, it is logical to postulate that their developmental dynamics follow a wave-like pattern in response to periodic endogenous surges of FSH. Until recently, reference to a follicular wave was limited to follicles ≥4 mm, based simply on the limit of resolution of existing ultrasound equipment. At the microscopic level, there is no morphologic distinction between mid- and late-stage antral follicles <4 mm and those ≥4 mm [5,6]. At the cellular level, both size categories of follicles not only express FSH receptors, but have a similar level of expression on a per granulosa cell basis (reviewed in [38]). The periodic emergence of waves of follicles ≥4 mm in response to periodic surges in blood FSH concentrations [17], and the consistency in the number of follicles ≥2 mm [39], or ≥3 mm [40] recruited into successive waves indicates that follicles may become progressively entrained to waves from the earliest stages of development.

6.2. Wave emergence and follicular dominance

The availability of new ultrasound scanners capable of resolving structures as small as 1 mm permitted a study designed to characterize the developmental pattern of 1–3 mm follicles in cattle, and to determine the stage at which the future dominant follicle first attains a size advantage among its cohorts [41]. Results revealed a change over days ($P < 0.05$) in the number of 1–3 mm follicles, with a maximum ($P < 0.05$) 1 or 2 days before conventionally defined wave emergence

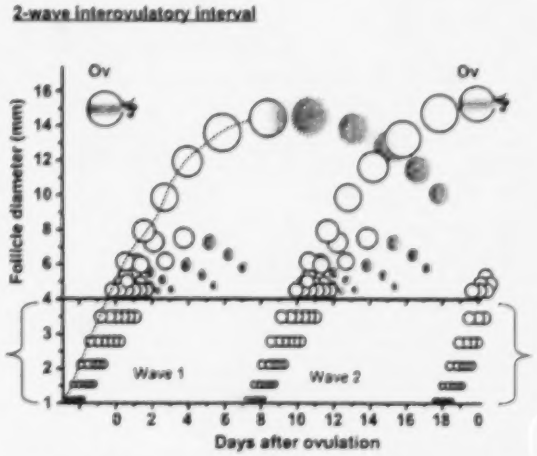


Fig. 2. Ovarian follicular wave pattern detected in follicles as small as 1 mm in diameter (two-wave pattern shown). Small follicles (1–3 mm) in parentheses illustrate wave emergence 2.5 days earlier than previously detected (i.e., at 4–5 mm). Note that the growth rate of the follicle destined to become dominant (dotted line) is similar to others in the wave until about 5 days after wave emergence (beginning at 1 mm), and that the follicle destined to become dominant has a size advantage over those destined to become subordinate at its earliest detection (1 mm).

(dominant follicle first detected at 4 mm), followed 3–4 days later by a maximum ($P < 0.05$) in the number of ≥4 mm follicles (Fig. 2). The future dominant follicle was first identified at a diameter of 1 mm and emerged 6–12 h earlier than the first subordinate follicle ($P < 0.01$; Fig. 2). After detection of the dominant follicle at 1 mm (0 h), its diameter was greater than that of the first and second subordinate follicles at 24 h ($P = 0.04$) and 12 h ($P = 0.01$), respectively, when the dominant follicle was 2.4 ± 0.17 mm and 1.7 ± 0.14 mm (Fig. 2). The growth rate of the dominant follicle was greater than that of the first and second subordinate follicles at 120 h ($P = 0.03$) and 108 h ($P = 0.02$), respectively, when the dominant follicle was 9.5 ± 0.30 mm and 8.8 ± 0.49 mm. The authors concluded that: (1) 1–3 mm follicles develop in a wave-like manner in association with surges in plasma concentrations of FSH; (2) 1–3 mm follicles are exquisitely responsive to transient elevations in FSH (i.e., within 6 h); (3) selection of the dominant follicle is manifest earlier than previously documented and is characterized by a hierarchical progression over a period encompassing the entire FSH surge (5 days).

7. Reproductive aging

Endocrine and ovarian characteristics of reproductive aging were characterized in a series of recent studies in which old cows (≥15 year) were compared with their young (≤5 year) daughters [42–44]. Mean circulating FSH concentrations were consistently higher in old cows than in their daughters, and the expected pattern of FSH secretion and wave emergence was maintained in old cows, i.e., each ovarian follicular wave was preceded by a surge in circulating FSH. Despite elevated FSH, fewer 4–5 mm follicles were recruited into each follicular wave in old cows than in their daughters. This interesting inverse relationship between the number of follicles recruited into a wave and the peak concentrations of FSH has also been reported in studies documenting the repeatability of follicles numbers within individuals [39,40].

The two-wave pattern occurred in 60% of the estrous cycles of old cows and the three-wave pattern occurred in the remainder, similar to their daughters [42]. The majority of mother–daughter pairs (six out of nine) had the same wave pattern. The length of interovulatory and interwave intervals did not change with age. The ovulatory follicle of old cows with a two-wave pattern was smaller at the time of ovulation than that of young cows. The diameter of the CL was smaller, and the plasma concentration of progesterone tended to be lower in old than in young cows. There was no age effect on circulating LH concentrations or LH pulse frequency. The emergence of an additional wave during the IOI resulted in greater follicular attrition in three- than in two-wave patterns [24], providing rationale for the hypothesis that depletion of the follicular reserve and onset of reproductive senescence may occur earlier in individuals exhibiting predominantly three- versus two-wave patterns.

The hypothesis that aging of the hypothalamo–pituitary axis in cattle is associated with a decrease in synchrony of the FSH surge and follicular wave emergence was tested in a recent study involving estradiol/progesterone-based ovarian synchronization [43]. Steroid treatment suppressed circulating FSH in both age groups for 36 h, and the intervals from treatment to subsequent FSH peak (3.7 ± 0.2 days) and wave emergence (4.3 ± 0.3 days) were not different between old and young cows. In a study of the ovarian response to superstimulatory treatment, fewer small (<5 mm) follicles were recruited into the follicular wave, and fewer 6–8 mm, 9–11 mm and ≥12 mm follicles developed after ovarian superstimulation in old cows than in their young daughters. On average, young cows had eight more ovulations than old cows.

Results of the latest in the series of studies between young and old cows [44] suggest that fertilization or cleavage rates decline with age. Fewer embryos and a higher proportion of unfertilized oocytes and/or uncleaved zygotes were recovered from old cows compared to their young daughters. This conclusion was supported by the observation that of the total oocytes and embryos recovered per donor, significantly more old cows (10/15; 67%) produced <50% embryos compared to their young daughters (4/16; 25%). The survival of embryos obtained from old cows and their daughters after transfer into young recipients did not differ. As well, pregnancy loss did not differ between embryos transferred from old versus young cows.

8. Summary and conclusions

Wave-like follicle development in cattle is manifest as simultaneous emergence of a group of small (i.e., 1 mm) follicles in both ovaries in response to a surge in circulating FSH concentrations. The largest of the group at its earliest detection (1 mm) usually remains dominant (random distribution in either left or right ovary), but individual follicle growth rates are similar until the dominant follicle reaches 8–9 mm (i.e., 5 days after emergence at 1 mm). Acquisition of LH receptors in granulosa cells of the dominant follicle results in

rapid divergence in growth rates; the dominant follicle continues to grow as the subordinates cease and begin to regress in a milieu of minimal FSH. Growing, static and regressing phases of dominant and subordinate follicles are morphologically distinct (based on histology and ultrasonography) and are reflective of functional status (steroid and protein metabolism and oocyte competence). The vast majority of estrous cycles are composed of two or three follicular waves. Two-wave cycles are consistently shorter (19–20 days) than three-wave cycles (22–23 days). The number of follicles recruited into each wave varies greatly among individuals, but is highly repeatable within individuals. The wave pattern tends to be repeatable within individuals, and duration of dominance of the first wave is predictive of the wave pattern. Reproductive aging in cattle is characterized by an elevation in plasma FSH concentrations, a decrease in the number of follicles recruited into each wave, a lower superovulatory response, and a lower oocyte fertilization rate.

Acknowledgements

Authors' research was supported by grants from the Natural Science and Engineering Research Council of Canada and the Saskatchewan Agricultural Development Fund. In-kind support from Bioniche Animal Health (Belleville, ON, Canada) is gratefully acknowledged.

References

[1] Cobb M. Generation: the seventeenth century scientists who unraveled the secrets of sex, life, and growth. NY, USA: Bloomsbury Publishing; 2006. p. 333.
[2] Smitz J, Cortvrindt R. The earliest stages of folliculogenesis in vitro. Reproduction 2002;123:185–202.
[3] Erickson BH. Development and radio-response of the prenatal bovine ovary. J Reprod Fertil 1966;10:97–105.
[4] Russe I. Oogenesis in cattle and sheep. Bibl Anat 1983;24:77–92.
[5] Braw-Tal R, Yossefi S. Studies in vivo and in vitro on the initiation of follicle growth in the bovine ovary. J Reprod Fertil 1997;109:165–71.
[6] Lussier JG, Matton P, Dufour JJ. Growth rates of follicles in the ovary of the cow. J Reprod Fertil 1987;81:301–7.
[7] Hammond J. The physiology of reproduction in the cow. Cambridge, London: Cambridge University Press; 1927; Br Vet Bull 1955;11:165.
[8] McNutt GW. The corpus luteum of pregnancy in the domestic cow (*Bos taurus*) and a brief discussion of cyclical ovarian changes. Am J Vet Med Assoc 1927;72:286–99.
[9] Cole HH. A study of the mucosa of the genital tract of the cow, with special reference to the cyclic changes. Am J Anat 1930;46:261–301.
[10] Bullough WS. Mitotic activity in the adult female mouse, *Mus musculus* L. A study of its relation to the oestrous cycle in normal and abnormal conditions. Philos Trans R Soc Lond Ser B: Biol Sci 1946;231:453–516.
[11] Mandle AM, Zuckerman S. Numbers of normal and atretic oocytes in unilaterally spayed rats. J Endocrinol 1950;6:426–35.
[12] Green SH, Zuckerman S. The number of oocytes in the mature rhesus monkey (*Macaca mulatta*). J Endocrinol 1951;7:194–202.
[13] Rajakoski E. The ovarian follicular system in sexually mature heifers with special reference to seasonal, cyclical, and left–right variations. Acta Endocrinol 1960;34:7–68.
[14] Adams GP. Comparative patterns of follicle development and selection in ruminants. J Reprod Fertil Suppl 1999;54:17–32.
[15] Singh J, Adams GP, Pierson RA. Promise of new imaging technologies for assessing ovarian function. Anim Reprod Sci 2003;78:371–99.
[16] Adams GP, Pierson RA. Bovine model for study of ovarian follicular dynamics in humans. Theriogenology 1995;43:113–20.
[17] Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. Association between surges of FSH and the emergence of follicular waves in heifers. J Reprod Fertil 1992;94:177–88.
[18] Bo GA, Adams GP, Pierson RA, Mapletoft RJ. Exogenous control of follicular wave emergence in cattle. Theriogenology 1995;43:31–40.
[19] Adams GP. Control of ovarian follicular wave dynamics in mature and prepubertal cattle for synchronization and superstimulation. In: Proceedings of the XX congress of the world association for buiatrics, vol. 2; 1998. p. 595–605.
[20] Ahmad N, Townsend EC, Dailey RA, Inskeep EK. Relationship of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. Anim Reprod Sci 1997;49:13–28.
[21] Bleach ECL, Glencross RG, Knight PG. Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. Reproduction 2004;127:621–9.
[22] Townson DH, Tsang PCW, Butler WR, Frajblat M, Griel Jr LC, Johnson CJ, et al. Relationship of fertility to ovarian follicular waves before breeding in dairy cows. J Anim Sci 2002;80:1053–8.
[23] Bo GA, Baruselli PS, Martinez MF. Pattern and manipulation of follicular development in *Bos indicus* cattle. Anim Reprod Sci 2003;78:307–26.
[24] Jaiswal RS, Singh J, Marshall L, Adams GP. Repeatability of 2- and 3-wave patterns during the bovine estrous cycle. Theriogenology; submitted for publication.
[25] Ginther OJ, Kastelic JP, Knopf L. Intraovarian relationships among dominant and subordinate follicles and the corpus luteum in heifers. Theriogenology 1989;32:787–95.
[26] Bergfelt DR, Lightfoot KC, Adams GP. Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. Theriogenology 1994;42:895–907.
[27] Singh J, Brogliatti GM, Christensen CR, Adams GP. Active immunization against follistatin and its effect on FSH, follicle development and superovulation in heifers. Theriogenology 1999;52:49–66.
[28] Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. Selection of the dominant follicle in cattle: role of two-way functional coupling between follicle-stimulating hormone and the follicles. Biol Reprod 2000;62:920–7.

[29] Jaiswal R. Regulation of follicular wave patterns in cattle. PhD Thesis. University of Saskatchewan; 2007. p. 169.
[30] Salamone DF, Adams GP, Mapletoft RJ. Changes in the cumulus–oocyte complex of subordinate follicles relative to follicular wave status in cattle. Theriogenology 1999;52:549–61.
[31] Vassena R, Mapletoft RJ, Allodi S, Singh J, Adams GP. Morphology and developmental competence of bovine oocytes relative to follicular status. Theriogenology 2003;60: 923–32.
[32] Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS, et al. Oocyte competence under different progestational environments. Biol Reprod 2006;109:166.
[33] Richards JS, Midgley AR. Protein hormone action: a key to understanding ovarian follicular and luteal cell development. Endocrinology 1976;98:929–34.
[34] Wandji SA, Fortier MA, Sirard MA. Differential response to gonadotrophins and prostaglandin E2 in ovarian tissue during prenatal and postnatal development in cattle. Biol Reprod 1992;46:1034–41.
[35] Itoh T, Kacchi M, Abe H, Sendai Y, Hoshi H. Growth, antrum formation, and estradiol production of bovine preanatral follicles cultured in a serum-free medium. Biol Reprod 2002;67: 1099–105.
[36] Fricke PM, Al-Hassan MJ, Roberts AJ, Reynolds LP, Redmer DA, Ford JJ. Effect of gonadotropin treatment on size, number, and cell proliferation of antral follicles in cows. Domest Anim Endocrinol 1997;14:171–80.
[37] Tanaka Y, Nakada K, Moriyoshi M, Sawamukai Y. Appearance and number of follicles and change in serum FSH in female bovine fetuses. Reproduction 2001;121:777–82.
[38] Bao B, Garverick HA. Expression of steroidogenic enzyme and gonadotropin receptor genes in bovine follicles during ovarian follicular waves: a review. J Anim Sci 1998;76:1903–21.
[39] Singh J, Dominguez M, Jaiswal R, Adams GP. A simple ultrasound test to predict superovulatory response in cattle. Theriogenology 2004;62:227–43.
[40] Burns DS, Jimenez-Krassel F, Ireland JL, Knight PG, Ireland JJ. Numbers of antral follicles during follicular waves in cattle: evidence for high variation among animals, very high repeatability in individuals, and an inverse association with serum follicle-stimulating hormone concentrations. Biol Reprod 2005;73:54–62.
[41] Jaiswal RS, Singh J, Adams GP. Developmental pattern of small antral follicles in the bovine ovary. Biol Reprod 2004;71:1244–51.
[42] Malhi PS, Adams GP, Singh J. Bovine model for the study of reproductive aging in women: follicular, luteal and endocrine characteristics. Biol Reprod 2005;73:45–53.
[43] Malhi PS, Adams GP, Pierson RA, Singh J. Bovine model of reproductive aging: response to ovarian synchronization and superstimulation. Theriogenology 2006;66:1257–66.
[44] Malhi PS, Adams GP, Mapletoft RJ, Singh J. Oocyte developmental competence in a bovine model of reproductive aging. Reproduction 2007;134:233–9.

Final Report – ADF Project # 20050731

OPTIMIZING FERTILITY AND OOCYTE QUALITY AFTER ARTIFICIAL INSEMINATION IN BEEF CATTLE

Published Abstracts and Presentations/Posters in Conference Proceeding

with Saskatchewan ADF Funding Support

Effect of short- versus long-proestrus on fertility in beef cattle after fixed-time artificial insemination

Dinesh Dadarwal, Reuben Mapletoft, Gregg Adams, Cody Creelman, Jaswant Singh
Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK Canada S7N 5B4

Selection and early growth of an ovulatory dominant follicle normally occurs in an environment of high-progesterone, while its final stages of growth (proestrus) occur in a low-progesterone environment. We tested the hypotheses that a) luteal-phase plasma progesterone exposure to growing dominant follicle followed by short duration of proestrus will result in lower fertility than longer proestrus, and b) low progesterone exposure during early growth of dominant follicle will overcome the effects of short proestrus. Heifers (n=61) and postpartum beef cows (n=80) were assigned randomly to four groups: high progesterone-long proestrus (HL), high progesterone-short proestrus (HS), low progesterone-long proestrus (LL) and low progesterone-short proestrus (LS). Cattle received two doses of prostaglandin F2α (PGF), 11 d apart, to synchronize the estrous cycle. Nine days later (Day 0), estradiol-17β and progesterone were given im to synchronize follicular wave emergence and a once-used CIDR device was inserted intravaginally to maintain low plasma progesterone concentrations. At the same time, low progesterone groups (LL & LS) were given two doses of PGF 12 h apart, while, high progesterone groups (HL & HS) were allowed to retain the corpus luteum (CL). The CIDR were removed on Day 7 (HL, LL) or Day 8 (HS, LS). All animals received PGF on the day of CIDR removal and pLH 12 h later to induce ovulation. Artificial insemination (AI) was done 12 h after the pLH injection. Transrectal ultrasound was used to measure the follicular diameter on the day of AI, CL diameter on Day 9.5 post-AI, and to perform pregnancy diagnosis 60 d post-AI. Follicle and CL diameter data were analyzed by ANOVA and pregnancy rates were compared by the logistic regression. Low progesterone and long proestrus resulted in higher pregnancy rates ($P<0.023$; 54.0%, 38.2%, 46.4% and 12.2%, in LL, LS, HL and HS groups, respectively). There was no interaction between levels of progesterone and duration of proestrus ($P=0.12$) and the pattern was similar in cows and heifers ($P=0.47$). The size of the preovulatory follicle was larger ($P=0.001$) in low progesterone groups than in high progesterone groups, and in cows than in heifers ($P=0.039$). The CL diameters on Day 9 post-AI were smaller and progesterone concentrations were lower in high-progesterone groups ($P=0.023$, $P=0.003$, respectively) and in the short-proestrus groups ($P=0.003$, $P=0.0002$, respectively) than in low-progesterone groups and long-proestrus groups.

In conclusion, a short proestrous interval resulted in a reduced pregnancy rate following fixed-time AI in beef cattle. Although a low progesterone environment during growth of the ovulatory follicle increased ovulatory follicle size and subsequent CL size and function, it did not compensate for the effect of shortened proestrus on pregnancy rates.

Key words: beef cows, heifers, fertility, proestrus, progesterone.

Acknowledgements: Supported by funding from Saskatchewan Agricultural Development Fund, Westgen Inc. and Natural Sciences and Engineering Research Council of Canada.

THE EFFECTS OF PROGESTERONE CONCENTRATION AND DURATION OF PROESTRUS ON FERTILITY IN BEEF CATTLE AFTER FIXED-TIME ARTIFICIAL INSEMINATION

Dadarwal D, Mapletoft RJ, Adams GP, Creelman C, Singh J
Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK Canada S7N 5B4

Objective: To determine the effect of altering circulating progesterone concentration during growth of ovulatory dominant follicle and the length of the proestrus period on fertility in beef cattle following fixed-time artificial insemination (FTAI).

Methods: Post-pubertal beef heifers (n=61) and postpartum beef cows (n=80) were assigned randomly to four groups: high progesterone-long proestrus (HL), high progesterone-short proestrus (HS), low progesterone-long proestrus (LL) and low progesterone-short proestrus (LS).Two doses of prostaglandin F2α (PGF), 11 d apart, were followed 9 d later (Day 0) by an injection of estradiol-17β and progesterone im and insertion of a once-used CIDR vaginal device. At the same time (i.e. Day 0), low progesterone groups (LL & LS) were given two doses of PGF 12 h apart. On Day 7 (HL, LL) or Day 8 (HS, LS), CIDR were removed and PGF was given. Porcine LH was given 12 h after CIDR removal and FTAI was done 12 h later. Transrectal ultrasound was used on the day of FTAI to measure preovulatory follicular diameter, 9.5 days after FTAI for CL diameter, and 60 d post-FTAI for pregnancy diagnosis. Follicle and CL diameter data were analyzed by ANOVA and pregnancy rates were compared by the logistic regression.

Results: Low progesterone and long proestrus resulted in higher pregnancy rates ($P<0.023$; 54.0%, 38.2%, 46.4% and 12.2%, in LL, LS, HL and HS groups, respectively) with no interaction between levels of progesterone and duration of proestrus ($P=0.12$). The pattern did not differ in cows versus heifers ($P=0.47$). The low progesterone groups had a larger preovulatory follicle than high progesterone groups ($P=0.001$). The CL diameters 9.5 d post-FTAI were smaller and progesterone concentrations were lower in high-progesterone groups ($P=0.023$, $P=0.003$, respectively) and in the short-proestrus groups ($P=0.003$, $P=0.0002$, respectively) than in low-progesterone groups and long-proestrus groups.

Conclusions: A longer duration of proestrus and low progesterone concentrations during ovulatory follicle growth increased pregnancy rate following FTAI in beef cattle. Despite increasing the preovulatory follicle size and subsequent CL size and function, a low progesterone environment during growth of ovulatory follicle did not compensate for the effect of the short proestrus on pregnancy rate.

EFEITOS DA PROGESTERONE EXÓGENA NO DESENVOLVIMENTO FOLICULAR E TAXA DE OVULAÇÃO EM NOVILHAS PRÉ-PUBERES

Pfeifer, L.F.M.[1,2]; Siqueira, L.G.B.[1]; Mapletoft, R.J.[1]; Adams, G.P.[1]; Singh; J.[1]

[1]University of Saskatchewan, Saskatoon, SK, Canada; [2]Universidade Federal de Pelotas, Departmento de Zootecnia, Pelotas, RS, Brazil; Campus Universitário, s/nº Caixa Postal 354 96010-900 Pelotas, RS

O tratamento de progesterona pode antecipar o início da puberdade em novilhas de corte. O uso de prostaglandina associado a progesterona pode aumentar a taxa de ovulação, já que a prostaglandina aumenta a liberação de GnRH novilhas pre-púberes (Lammoglia, MA., Theriogenology, 1995). Os objetivos deste estudo foram determinar os efeitos de progesterona exógena associada ou não à prostaglandina, no desenvolvimento folicular e taxa de ovulação em novilhas proximas a atingir a puberdade. Novilhas Hereford (n=38), 10 a 12 meses de idade e peso vivo entre 255 e 320 Kg, foram examinadas duas vezes em um intervalo de 10 dias, por ultrasonografia transretal para confirmar que não eram púberes (i.e., ausência de CL). Os animais foram alocados aleatoriamente em três grupos distintos e receberam: 1) progesterona (grupo P, n=13), 2) progesterona e prostaglandina F2α (grupo PPG, n=11), ou sem tratamento (grupo Controle, n=14). Um implante intravaginal impregnado com progesterona (CIDR®) foi inserido nas novilhas dos grupos P e PPG em estágios aleatórios ddo ciclo estral, enquanto animais do grupo controle não receberam nenhum tipo de tratamento. Os ovários de todas as novilhas foram examinados diariamente por ultrasonografia transretal a partir do dia da inserção do CIDR para monitorar a dinâmica folicular e ovulação. O dia de emergência da onda folicular após a inserção do CIDR foi registrado e o CIDR removido 5 dias após. O grupo PPG recebeu uma injeção i.m. de 500 µg de cloprostenol (análogo da PGF2α) no dia da remoção do CIDR. A dinâmica folicular foi monitorada por 5 dias após remoção do CIDR nos grupos P e PPG, ou até a detecção de um folículo dominante de 8mm oriundo da próxima onda folicular no grupo Controle. A taxa de ovulação foi comparada entre grupos pelo teste do qui-quadrado e a dinâmica folicular por análise de variância para medidas repetidas (procMIXED, SAS). A proporção de novilhas que ovularam foi alta no grupo PPG (72.7%, 8 de 11), intermediária no grupo P (30.8%, 4 de 13) e baixa no grupo controle (7.1%, 1 de 14; P<0.001). A taxa de ovulação foi maior nos grupos que receberam progesterona (grupos P e PPG combinados) obtiveram (50%, 12 de 24) comparada ao controle (P<0.01). O diâmetro do folículo dominante no dia 7 foi maior em novilhas tratadas com progesterona do que no grupo controle (12,5±0,25 mm, 12,72±0,34 mm and 11,45±0,25 mm, para os grupos P, PPG e Controle, respectivamente; P<0,01). A exposição a progesterona em novilhas próximas a puberdade está associada a maior crescimento do folículo dominante após o tratamento e maior taxa de ovulação, particularmente quando e aplicada prostaglandina após tratamento com progesterona. O tratamento combinando progesterona e prostaglandina mostrou-se uma ferramenta importante para antecipar a puberdade em novilhas pre-púberes, o que facilmente pode ser aplicado aos sistemas de produção animal.

EFFECT OF EXOGENOUS PROGESTERONE ON FOLLICULAR DEVELOPMENT AND OVULATION RATE IN PREPUBERTAL HEIFERS

Progesterone treatment can hasten the onset of puberty in prepubertal heifers. The use of prostaglandin associated with progesterone treatment can increase ovulation rate, since prostaglandin stimulate the releasing of GnRH in prepubertal heifers (Lammoglia, MA., Theriogenology, 1995). The objectives of this study were to determine the effects of exogenous progesterone associated or not to PGF2α on follicular development and ovulation rate in near-pubertal heifers. Hereford heifers (n=38), 10 to 12 months of age and weighed 255 – 320 Kg, were examined twice, 10 days apart, by transrectal ultrasonography to confirm that they were not yet pubertal (i.e., the absence of a CL). The heifers were assigned randomly to three groups and given 1) progesterone (P group, n= 13), 2) progesterone plus prostaglandin F2α (PPG group, n= 11), or no treatment (Control group, n= 14). An intravaginal progesterone-releasing device (CIDR®) was inserted into heifers in the P and PPG groups at random stages of the follicular wave, while controls received no treatment. The ovaries of all heifers were examined daily by transrectal ultrasonography from the day of CIDR insertion to monitor follicular dynamics and ovulation. The day of follicular wave emergence after CIDR insertion was recorded and CIDR were removed 5 days later. The PPG group received 500 µg cloprostenol (PGF2α analog) im on the day of CIDR removal. Follicular dynamics were monitored for 5 days after CIDR removal in P and PPG groups, or until the detection of an 8 mm dominant follicle of the subsequent follicular wave in the Control group. The incidence of ovulation was compared among groups by chi-square analysis and follicular diameter profiles by analysis of variance for repeated measures (procMIXED, SAS). The proportion of heifers ovulating was highest in the PPG group (72.7%, 8 of 11), intermediate in the P group (30.8%, 4 of 13), and lowest in the control group (7.1%, 1 of 14; P<0.001). Overall, progesterone treatment (P and PPG groups combined) resulted in a higher incidence of ovulation (50%, 12 of 24) compared to controls (P<0.01). The diameter of the dominant follicle on Day 7 was larger in progesterone-treated heifers than in the control group (12.5±0.25 mm, 12.72±0.34 mm and 11.45±0.25 mm, for P, PPG and Control groups, respectively; P<0.01). In summary, progesterone exposure and withdrawal in near-pubertal heifers was associated with greater growth of the post-treatment dominant follicle and an increased capacity for it to ovulate, particularly in combination with post-progesterone prostaglandin treatment. The combination of progesterone and prostaglandin treatments showed to be valuable in hasten the onset of puberty in beef heifers, which easily can be applicable for livestock production.

Effect of short versus long proestrous period along with low or high progesterone concentrations during the ovulatory wave on beef cattle fertility after FTAI

C. Creelman, D. Dadarwal, J. Singh

Veterinary Biomedical Sciences, Western College of Veterinary Medicine, 52 Campus Drive, Saskatoon, Saskatchewan

Introduction

The indirect endocrine effects of progesterone during follicular development and maturation play a role in oocyte competence and subsequent fertility

wave or 3-wave interovulatory intervals

Follicles grown in 2-wave vs. 3-wave are exposed to high progesterone for 5 days vs. 3 days and have a diminished fertility

Superstimulation of cattle ovaries produces oocytes exposed to similar preovulatory environments as those in 2-wave interovulatory interval

Objective and Hypothesis

Manipulate the proestrus of cattle into short and long durations, in order to see its effects on fertility

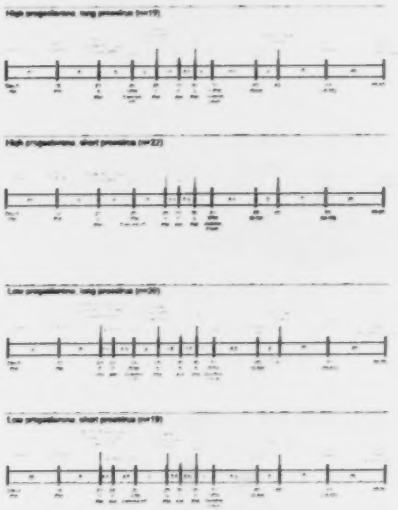
Lowering progesterone concentrations during oocyte's growth will increase fertility

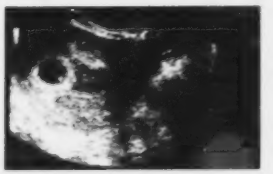
:Fertility in

- the long proestrus groups (high or low progesterone) will be similar
- the short proestrus/high progesterone group will be reduced as compared to all groups
- the short proestrus/low progesterone group will be similar to both long proestrus groups

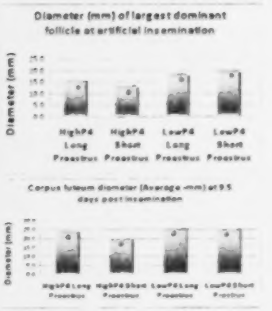
Materials & Methods

Experimentation was conducted on Hereford cattle (n=80) at the University of Saskatchewan's Gooddale Research Farm





Results


Diameter (mm) of largest dominant follicle at artificial insemination
Diameter (mm)
HighP4 Long Proestrus
HighP4 Short Proestrus
LowP4 Long Proestrus
LowP4 Short Proestrus
Corpus luteum diameter (Average -mm) at 9.5 days post insemination
Diameter (mm)

Diameter of dominate follicle (mm) at artificial insemination showed a significant increase based on lower progesterone concentrations in both)proestrus groups (P<0.001

Corpus Luteum diameter (mm) at 9.5 days post insemination was lowest in high progesterone/short proestrus)group than all other groups. (P<0.05

Pregnancy rates at 60 days differed significantly between high progesterone/short proestrus and low progesterone/long proestrus groups (P<0.05)

Pregnancy rates at 60 days approached significant difference between high progesterone/long proestrus and high progesterone/short)proestrus groups (P=0.0517

Conclusion

Lowering progesterone results in a larger dominant follicle in both proestrus groups

R
reduced as a result of both short proestrus and high progesterone

The hypothesis, that artificially lowering progesterone concentrations during a short proestrus will increase pregnancy rates was not supported, however there was a numerical .increase

Further indicators of oocyte competence such as / fertilization and blastocyst rates should be evaluated in the future

Acknowledgements

I would like to thank the WCVM, The Department of Veterinary Biomedical Sciences, Druvtej Ambati, Rand Davis and the rest of the "Team Repro". As well as the Interprovincial Research Scholarship program.

Botu-Bov extenders. Eighteen ejaculates of bulls from Alta Genetics – Brazil were divided in two fractions and each one was frozen either with Andromed (Minitub) or Botu-Bov (Biotech Botucatu) extenders, according to the manufacturer recommendation. Andromed: initial dilution 1:1 (v:v), 10 minutes at 37°C, dilution again until final concentration, maintenance for 1 hour at 18°C, packing in 0.25mL straws, stabilizing at 5°C for 4 hours and freezing in the machine in a curve of -30°C/minute. Botu-Bov: total dilution at room temperature, packing in 0.25mL straws, stabilizing for 4 hours at 5°C and freezing by the same method. Straws were thawed at 37°C for 30 second in water bath, then maintained in dry block at 37°C for 4 hours. Motility characteristics were evaluated at 10 minutes and 4 hours after thawing by CASA (IVOS 12). Integrity of plasma and acrosomal membranes was evaluated 10 minutes after thawing by epifluorescence with Propidium Iodide, FITC-PSA and JC1. At 10 minutes after thawing, Botu-Bov extender was statistically superior (P<0.001) to Andromed in total motility (86 vs 80%), plasma membrane integrity (57 vs 35%), linearity (80 vs 69%) and straightness (52 vs 40%). There was no difference (P>0.05) between extenders in progressive motility and acrosomal integrity at this moment. At 4 hours after thawing, there were no differences (P>0.05) in total motility (57 vs 64%), progressive motility (25 vs 30%) and linearity (67 vs 75%) between Botu-Bov and Andromed, respectively, except for straightness (42 vs 50%, P<0.01). Botu-Bov extender present higher sperm viability immediately post-thawing, although this difference is not verified after a period of 4 hours. On the other hand, the procedure for semen freezing with Botu-Bov is more practical, which makes it easier to use in farm conditions. Fertility trials are necessary to verify *in vivo* effects of these extenders.

P029

Effect of length of progesterone exposure during the growing phase of the ovulatory follicles on oocyte competence after superstimulatory treatment

Dias, F[1*], Costa, E[1], Adams, GP[1], Mapletoft, RJ[2], Dochi, O[3], Singh, J[1]
[1]*Veterinary Biomedical Science, University of Saskatchewan, Canada;* [2]*Large Animal Clinical Sciences, University of Saskatchewan, Canada;* [3]*Rakuno Gakuen University, Japan*

The objective was to determine the effect of the duration of progesterone influence during the growing phase of pre-ovulatory follicles on oocyte competence after superstimulatory treatment. We tested the hypotheses that a short period of progesterone (P4) exposure improves oocyte competence, and that FSH starvation at the end of superstimulatory treatment does not affect oocyte competence. Forty cross-breed beef cows (weight, 515 to 795 kg) were allocated randomly to three groups (short P4, FSH-starvation, and long P4 group). To synchronize wave emergence and standardize the luteal phase among animals, transvaginal ultrasound-guided ablation of follicles ≥ 5mm was done 5 to 8 days after ovulation and CIDR (Pfizer Animal Health) was placed intravaginally. Cows in short-P4 and FSH-starvation groups received eight injections of FSH (Folltropin-V; Bioniche Animal Health) at 12-hour intervals, whereas the long-P4 group received 14 injections of FSH, starting on the day of wave emergence (Day 0). The short-P4 group were given 25 mg of PGF twice, (12 hours apart), on Day 3, whereas both the FSH-starvation and long-P4 groups received PGF on Day 6. The CIDR were removed at the time of second PGF treatment. Cows were given LH (25 mg Lutropin-V, im; Bioniche Animal Health) 24 hours after CIDR removal and inseminated 24 and 36 hours later. Reproductive tracts were collected at the time of slaughter 4 days after insemination. In the FSH-starvation group, 6 out of 13, and in the long-P4 group, 1 out of 13 failed to ovulate and were excluded from further analysis. Fewer ovulations (number of CL at slaughter) were detected in the FSH-starvation group than both the short-P4 and long-P4 groups (mean±SEM: 4.6±2.0, 11.6±2.2, and 16.7±3.0, respectively; P=0.02). The number of ovulations did not differ between short-P4 and long-P4 groups. Ova-embryo recovery rate had a strong tendency to differ among groups (1.7±1.1, 5.9±1.3, and 7.3±1.6; P=0.07). A greater proportion of unfertilized ova were collected from cows in the short-P4 group than in the FSH-starvation and long-P4 groups (13/82, 0/12, and 5/87 in short-P4, FSH-starvation and long-P4 groups, respectively; P<0.03). In conclusion, the hypothesis that a short period of progesterone exposure improves oocyte competence was not supported. The hypothesis that FSH starvation at the end of superstimulatory treatment does not affect oocyte competence was supported; however, the FSH-starvation treatment used in this experiment led to a loss of ovulatory capability.
Research supported by NSERC & ADF.

P030

Effects of by-pass fat feeding on the reproductive performance of first-calf Brahman cows under tropical sabana conditions

Diaz, T[1*], Betancourt, R[2], Hernandez, R[2], Gallo, J[3]
[1]*Animal Reproduction Institute, Facultad de Ciencias Veterinarias, UCV, Venezuela;* [2]*Animal Nutrition, Facultad de Ciencias Veterinarias, UCV, Venezuela;* [3]*Animal Nutrition, Universidad de Antioquia, Colombia*

First-calf beef cows located on tropical areas of the world are under a high degree of stress due to climatic conditions and a poor nutritional status. Bypass fat feeding decreases the negative energetic balance of the postpartum cow and improves the reproductive performance. To test the effect of bypass fat feeding as an alternative to decrease the negative energy balance, 27 first-calf cows, under savannah conditions, were divided into 3 groups, according to the number of days open: group 1 (n=10; G1), cows with a mean of 100+8d open; group 2 (n=9; G2), cows with 165+6d open in average and group 3 (n=8; G3) with 233+5d open. Cows were nursing a calf. Fat feeding started 85d before the onset of the breeding season (BS) under natural mating. Experiment was done during the dry-rainy transition period. Nutritional supplement contained 20% of by-pass fat (17% of linoleic acid) and was made to allow a consumption of 500g/d. Body weight (BW), body condition score (BCS; 1-9), cholesterol (CHOL), triglycerides were measured every month until the beginning of a 4 months BS, then every 2 months until the end of BS. Ultrasound scanning was performed at onset, middle and end of BS, to verify luteal activity (onset) and for pregnancy diagnosis (middle, end). Pasture (Brachiaria humidicola) quality and biomass availability tests were performed. Data were analyzed by ANOVA using SAS procedures. Cows in G1, G2 and G3 had BCS and BW 5+.4; 5.4+.4 and 5.1+.2; 349.3+26.1; 366+42.4 and 343.9+19.8 kg, respectively, at onset of BS. Fifty percent of cows in G1 had a mean area of luteal tissue (LT) of 1.56+.4 cm2, 66.5% of cows in G2 had 1.39+.7 cm2 of LT, whereas 75% in G3 had 1.85+.6 cm2 (P>.001). Pregnancy diagnosis 2 months from the beginning of the BS, showed that cows in G1, G2 and G3 had 30, 78 and 75% of PR (P<.07), respectively. On day 60 of BS 86% of open cows in G1 had an average of 1.7+.3 cm2 of LT; whereas in G2 and G3, 50% and 100% of open cows had 1.54 cm2 and 2.1+.4 cm2 of LT, respectively. Sixty days after onset of the BS, cows had 224 kg/Ha of forage availability, and forage had 5% of crude protein. At the end of the BS, G1, G2 and G3 had 70, 100 and 100% of PR (P>.1). In summary, 89.9% (24/27) of the cows were pregnant at the end on the BS. Mean levels of triglycerides, in all cows, decreased from 263+153.6 on d 0 to 124.7+31.2 mg/dL on d 90; whereas CHOL increased from 48.2+34.7 to 334.7+63.4 mg/dL, on the same days. In conclusion, feeding by-pass fat to first-calf beef cows could be an excellent alternative in tropical areas of the world to increase PR.

P031

The use of reduced doses of Dinoprost Trometamita in combination with intravaginal device with progesterone in order to control the oestrous cycle in beef cattle

Verellen, M[1*], Sarramone, C[2], Dick, A[1*] and Calejas, S[1]
[1]*Faculty of Veterinary Sciences, Tandil, U.N.C.P.B.A., Buenos Aires, Argentina;* [2]*Private activity*

The objective of this trial was to evaluate the effect of dose of Dinoprost trometamina (Lutalyse®, Pfizer Animal Health), administered at the end of a treatment to control the oestrous cycle

UNIVERSITY OF SASKATCHEWAN

YEARS *Engage · Enlighten · Explore*

Reproductive Science and Medicine Research Symposium

February 29th 2008

Span Room, Atrium Building, Innovation Place

THE EFFECT OF PROGESTERONE ON FOLLICULAR DEVELOPMENT AND PREGNANCY RATE IN BEEF CATTLE

Pfeifer LF[1,2], Mapletoft RJ[1], Adams GP[1], Kastelic J[4], Small J[3], Dias F[1], Singh J[1]

Western College of Veterinary Medicine, University of Saskatchewan[1], 52 Campus Drive, Saskatoon, Saskatchewan, Universidade Federal de Pelotas[2], Department of Animal Production, Brasil, Agriculture and Agri-food Canada[3], Research Centre, Brandon, Manitoba, Agriculture and Agri-Food Canada[4], Lethbridge Research Centre, Box 3000, Lethbridge, Alberta.

Objective: The objective of this study was to determine the effect of level of progesterone (P4) during the ovulatory wave on fertility.

Methods: Low versus high progesterone concentrations which were expected to result in high versus low LH pulse-frequency were compared. Lactating, postpartum beef cows (n=129) at random stages of the estrous cycle were given cloprostenol (PGF; Schering-Plough Animal Health, Pointe-Claire, QC, Canada) twice at an 11-day interval to synchronize ovulation. Cows were given 1.5 mg estradiol benzoate on Day 0 (D0). On Day 0, the Low-P4 group was given PGF and an intravaginal Cue-mate (Bioniche Animal Health, Belleville, ON, Canada) containing one P4 pod (expected to achieve P4 plasma level of 1 ng/ml). The High-P4 group also received a Cue-mate with one pod on day 0, but no PGF was administered (i.e., cows were allowed to retain their CL for 4 days after expected emergence of the estradiol-induced wave). On Day 8, Cue-mates were removed and PGF was administered to all animals. Fifty-four to 56 hr later, all animals received 12.5 mg of Lutropin-V (Bioniche) i/m and were timed-inseminated (TAI). Dominant follicle diameter was measured on Day 8 and at the time of AI. Pregnancy diagnosis was done by ultrasonography 28 days after AI to compare fertility between groups. Follicle diameter data were analyzed by variance analyses - ANOVA and pregnancy rate data were compared by the logistic regression.

Results: The dominant follicle in Low-P4 group was larger ($p<0.001$) than in high-P4 group on the day of the Cue-mate removal (Day 8) and on the day of the TAI (12.33 ± 0.34 and 14.27 ± 0.51 versus 14.18 ± 0.34 and 16.52 ± 0.52) in the High-P4 and Low-P4 group, respectively. The Low-P4 group showed larger CL, 24.68 ± 0.69 mm, than High-P4 group, 22.23 ± 0.76 mm ($p<0.002$). Pregnancy rate was similar ($p>0.05$) between the groups (High-P4: 57.38% (35/61) and Low-P4: 51.47% (35/68).

Conclusions: Low-P4 resulted in larger dominant follicle and larger CL at day 7 after AI, without any apparent effect on pregnancy rate.

PROCEEDINGS OF THE ANNUAL CONFERENCE OF THE INTERNATIONAL EMBRYO TRANSFER SOCIETY

Denver, Colorado

January 5–9, 2008

Main Theme: Needs

Edited on behalf of the Society by

Program Co-Chairs
Pat Lonergan
University College Dublin
Newcastle, County Dublin, Ireland

George E. Seidel, Jr.
Colorado State University
Fort Collins, CO, USA

Board of Governors 2007–2008

Naida Loskutoff, President
Takashi Nagai, Immediate Past President
Richard Fayrer-Hosken, Vice President
Richard Fayrer-Hosken, Secretary-Treasurer

Peter Farin, Governor
Rebecca Krisher, Governor
Pat Lonergan, Governor
Ed Squires, Governor
Ann Van Soom, Governor
Christine Wrenzycki, Governor

International Embryo Transfer Society
Jennifer Gavel, Executive Secretary
1111 North Dunlap Avenue
Savoy, IL 61874, USA

19 THE EFFECT OF PROGESTERONE ON FOLLICULAR DEVELOPMENT AND PREGNANCY RATE IN BEEF CATTLE

L. F. Pfeifer[A,B], *R. Mapletoft*[A], *G. Adams*[A], *J. Kastelic*[A], *J. Small*[A], *F. Dias*[A], *and J. Singh*[A]

[A]University of Saskatchewan, Saskatoon, SK, Canada;
[B]Universidade Federal de Pelotas, Pelotas, Rio Grande do Sul, Brasil

The objective was to determine the effect of the level of progesterone (P4) during the ovulatory wave on fertility. Low *v.* high progesterone concentrations which were expected to result in high *v.* low LH pulse-frequency were compared. Lactating, postpartum beef cows ($n = 69$) at random stages of the estrous cycle were given cloprostenol (PGF; Schering-Plough Animal Health, Pointe-Claire, QC, Canada) twice at an 11-day interval to synchronize ovulation (Day 0 = day of 2nd PGF). Cows were given 1.5 mg estradiol benzoate on Day 10 (7 days after expected ovulation). On Day 10, the Low-P4 group was given PGF and an intravaginal CueMate (Bioniche Animal Health, Belleville, ON, Canada) containing one P4 pod (expected to achieve a P4 plasma level of 1 ng mL^{-1}). The High-P4 group also received a CueMate with one pod on Day 10, but no PGF was administered (i.e., cows were allowed to retain their CL for 4 days after expected emergence of the estradiol-induced wave). On Day 18, CueMates were removed and PGF was administered to both groups. Fifty-four to 56 h later, all animals received 12.5 mg of Lutropin-V (Bioniche) IM and were timed AI (TAI). Dominant follicle diameter was measured on Day 18 and at the time of AI. Pregnancy diagnosis was done by ultrasonography 27 days after AI to compare fertility between groups. Follicle diameter data were analyzed by Student's *t*-test and pregnancy rate data were compared by the chi-square test. The dominant follicle in the Low-P4 group was larger ($P < 0.05$) than in the high-P4 group on the day of the CueMate removal (Day 18) and on the day of the TAI (11.8 ± 0.4 and 13.7 ± 0.5 *v.* 13.3 ± 0.6 and 15.3 ± 0.6 in the High-P4 and Low-P4 groups, respectively). Pregnancy rate was similar ($P = 0.16$) between the groups (High-P4: 55.6% (20/36) and Low-P4: 44.4% (16/33)); however, a larger study is in progress to confirm these results. To conclude, Low-P4 resulted in a larger dominant follicle without any apparent effect on pregnancy rate.

The project was funded by Saskatchewan ADF and NSERC. L. Pfeifer is funded through a CAPES scholarship. We acknowledge Schering-Plough and Bioniche for donation of pharmaceuticals.

20 ADMINISTRATION OF FOLLICLE-STIMULATING HORMONE AT CIDR REMOVAL DOES NOT AFFECT THE PREGNANCY RATE IN A CIDR-BASED, COSYNCH PROTOCOL IN LACTATING BEEF COWS

J. Small[A], *F. Dias*[B], *L. Pfeifer*[B], *K. Lightfoot*[C], *M. Colazo*[D], *J. Kastelic*[E], *and R. Mapletoft*[B]

[A]Agriculture and Agri-Food Canada, Brandon, MB, Canada;
[B]University of Saskatchewan, Saskatoon, SK, Canada;
[C]Assiniboia Veterinary Clinic, Assiniboia, SK, Canada;
[D]Alberta Agriculture and Food, Edmonton, AB, Canada;
[E]Agriculture and Agri-Food Canada, Lethbridge, AB, Canada

In previous studies, giving eCG at CIDR removal significantly increased the pregnancy rate after timed-AI in beef cows. However, eCG is not universally available. Therefore, we tested the hypothesis that giving pFSH at CIDR removal might improve the pregnancy rate in a CIDR-based, Cosynch protocol in postpartum, suckled *Bos taurus* beef cows; a secondary objective was to compare pregnancy rates when GnRH *v.* pLH was used to synchronize wave emergence and ovulation. This work was conducted as two experiments (separate locations). All cows were given a CIDR (containing 1.9 g progesterone; Pfizer Animal Health, Montreal, QC, Canada) on Day 0 (without regard to stage of estrous cycle or cyclicity). On Day 7, CIDRs were removed, all cows were concurrently given 25 mg PGF (Lutalyse; Pfizer Animal Health, Groton, CT, USA), half were given 20 mg pFSH (Folltropin-V; Bioniche Animal Health, Belleville, ON, Canada), and all cows were timed-AI 54 h later (Day 9). In Experiment 1, 240 cows [94 ± 10.8 days postpartum; body condition score (BCS: 1 = emaciated, 9 = obese; mean ± SD): 5.8 ± 0.4] were used; at CIDR insertion and AI, cows were allocated to receive either 100 μg GnRH ($n = 160$; Cystorelin, Merial Canada Inc., Victoriaville, QC, Canada) or 12.5 mg pLH ($n = 80$; Lutropin-V, Bioniche Animal Health). In Experiment 2, 109 cows (59.2 ± 19.5 days postpartum; BCS: 5.6 ± 1.1) were used; all received 100 μg GnRH (Cystorelin) at CIDR insertion and AI. In Experiment 1, three cows that lost their CIDR were excluded; pregnancy rates after timed-AI (logistic regression, backward selection: parity, pFSH, synchronizing treatment, and their interactions) were not different between cows given pFSH *v.* control cows (64.7 *v.* 65.2%; $P > 0.80$), nor between cows given GnRH *v.* pLH (62.7 *v.* 69.6%; $P = 0.91$). However, there was an interaction ($P < 0.04$) between parity and the synchronizing treatment; in primiparous cows, pregnancy rates were significantly lower in those given GnRH *v.* pLH (59.3 *v.* 83.3%). In Experiment 2, pregnancy rates after timed-AI (logistic regression, backward selection: parity, pFSH, and their interaction) were not different between cows given pFSH *v.* control cows (38.2 *v.* 42.6%; $P > 0.6$). In conclusion, our hypothesis was not supported; giving pFSH at CIDR removal did not significantly improve the pregnancy rate in a CIDR-based, Cosynch protocol in postpartum, suckled *Bos taurus* beef cows.

21 DEVELOPMENT OF A TIMED INSEMINATION PROGRAM IN DAIRY HEIFERS AS A PLATFORM TO DETERMINE IF FLUNIXIN MEGLUMINE IMPROVES PREGNANCY RATE AND EMBRYO SURVIVAL

W. W. Thatcher, C. A. Risco, J. Larson, M. J. Thatcher, F. Lima, and S. A. Woodall

University of Florida, Gainesville, FL, USA

The objectives of the present series of experiments were to develop a timed artificial insemination (TAI) program for dairy heifers and to utilize a TAI program to evaluate the effect of flunixin meglumine (Banamine®; Schering-Plough Animal Health Corp., Atlanta, GA, USA), a prostaglandin

Proceedings of the 53rd Annual Meeting of the Canadian Fertility and Andrology Society (2007)

Title: EFFECT OF LENGTH OF PROGESTERONE EXPOSURE DURING OVULATORY WAVE DEVELOPMENT ON PREGNANCY RATE

Dias F.C.F. [1], Stover B. C. [1], Mapletoft R. J. [2], Kastelic J.[3], Adams G. P. [1], Colazo M.[1], Dochi O.[1], Singh J. [1]

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine,
University of Saskatchewan, 52 Campus Drive, Saskatoon,
[2]Department of Large Animal Clinic, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon.
Saskatchewan, Canada S7N 5B4
[3]Agriculture and Agri-Food Canada, Lethbridge Research Centre, Box 3000, Lethbridge, AB, Canada T1J 4B1.

Objectives: The objective of this study was to determine the effect of length of the ovulatory wave on fertility (pregnancy rate). We tested the hypothesis that short period of progesterone exposure during the growing and early-static phase of the dominant follicle is associated with higher fertility.
Study Methods: Heifers (n=172) and post-partum cows (n=193) received estradiol-progesterone injection and CIDR on day 3-5 of the cycle to induce a new follicular wave. The short and long progesterone groups (analogous to ovulatory wave of 3-wave versus 2-wave estrous cycles) were given prostaglandin injection on Day 3 and 6 after wave emergence, respectively. CIDR implants were removed at this time and all animals received LH and were artificial inseminated 55hr later. Follicle and CL size were measured by transrectal ultrasonography at the time of CIDR removal, artificial insemination and 36 hours post AI. Pregnancy diagnosis was performed 30-35 days. Chi square analysis was used to determine the effect of short and long treatments on pregnancy rates. Follicle size was compared by 2-way ANOVA analysis.
Results: No difference in pregnancy rates were detected between short and long treatment groups in heifers (53% vs 47%, p=0.44) or cows (63% vs. 58%, p=0.51). The size of ovulatory follicle at the time of CIDR removal and AI was larger in long progesterone groups than in short progesterone groups (P=0.001; P=0.01). The size of ovulatory follicle at the time of CIDR removal and AI was larger in cows than heifers (P=0.001).
Conclusions: To conclude, results do not support our hypothesis. Ovulatory follicles grow to a larger size when the growth phase of the dominant follicle is extended by 3 days but there is no difference in the oocyte competence in estrous cycles with 2- vs. 3-follicular waves.

The 2007 Merck-Merial Symposium, USA

Ovulation Induction in Peri-pubertal Heifers: Follicular Dynamics and Endocrine Changes

*Chaîné K, Pfeifer LFM, Mapletoft RJ, Kastelic J, Colazo M and Singh J

Department of Biomedical Sciences, Western College of Veterinary Medicine
University of Saskatchewan, 52 Campus Drive, Saskatoon,
Saskatchewan, Canada, S7N 5B4

The objectives were to induce ovulation in peri-pubertal beef heifers (for early breeding) and to study ovarian follicular dynamics, corpus luteum life span, and plasma hormone concentrations after ovulation induction.

We tested the hypotheses that: 1) exogenous progesterone treatment during the growth phase of the dominant follicle, followed by progesterone withdrawal, will induce an LH peak and ovulation in peri-pubertal heifers; 2) the dominant follicle will grow at a slower rate in progesterone-treated versus control heifers; and 3) wave-synchronization followed by prostaglandin treatment (without progesterone treatment) will not result in ovulation (in control heifers).

Peri-pubertal Hereford heifers (n=22, 12-15 months of age, 305±5.8 kg body weight, uterine diameter 17.5±0.4 mm), were used. In these heifers, no corpus luteum was detected at two ultrasound examinations (7 days apart). On Day 0 (without regard to the stage of the ovarian follicular wave), 11 heifers were given 1 mg estradiol benzoate (in canola oil) im and an intravaginal progesterone releasing insert (CIDR-B; containing 1.9 g progesterone). On Day 7, the CIDR inserts were removed and heifers were concurrently given 500 µg cloprostenol im (to induce luteal regression). In the Control heifers (n=11), follicular wave emergence was induced on Day 3 by ultrasound-guided ablation of all follicles >5 mm. Daily ultrasound examinations were performed and blood samples were collected from Day 0 to ovulation (or to Day 12 in the absence of ovulation).

In the progesterone-treated group, 3 and 5 heifers ovulated on Days 10 and 11, respectively, whereas in the Control group, 2, 3, 2, and 1 heifers ovulated on Days 7, 10, 11 and 12. Largest diameter of the ovulatory follicle was not different (p=0.8) between treated and control heifers (13.3±0.6 versus 13.0±0.9 mm; mean±SD/SEM). Hormonal analyses are in progess. In conclusion, ovulation did occur in both treated and control groups and hypotheses 2 and 3 were not supported.

Project supported by research grants from the Saskatchewan Agriculture Development Fund and Westgen. K. Chaîné is supported by a Merck scholarship and Pfeifer LFM by a CAPES scholarship.

Effect of Length of Progesterone Exposure during Ovulatory Wave Development on Pregnancy Rate in Cattle

Fernanda C. Dias, Bruce C. Stover, Reuben J. Mapletoft, John P. Kastelic, Gregg P. Adams, Marcos Colazo, Osamu Dochi, Jaswant Singh

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine
University of Saskatchewan, Canada S7N 5B4

WCVM

Abstract

The objective of this study was to determine the effect of length of the ovulatory wave on fertility (pregnancy rate) in cattle. We tested the hypothesis that a short period of progesterone exposure during the growing and early-static phase of the dominant follicle is associated with higher fertility. Heifers (n=40) and post-partum cows (n=116) were given estradiol-progesterone treatment and a CIDR on day 3-5 after ovulation to induce a new follicular wave. Short- and long-progesterone groups (analogous to the ovulatory wave of 3-wave versus 2-wave estrous cycles) were given prostaglandin on Day 3 and 6 after wave emergence, respectively. CIDR implants were removed at the time of prostaglandin treatment, and all animals received LH and were artificial inseminated (AI) 55 hr later. Follicle and CL size were measured by transrectal ultrasonography at the time of CIDR removal, AI, and at 36 hours post-AI. Pregnancy diagnosis was performed 30-35 days after AI. Chi square analysis was used to compare pregnancy rates. Follicle size was compared by 2-way ANOVA. No difference in pregnancy rates were detected between short- and long-treatment groups in heifers (70% vs 45%, p=0.11) or cows (66% vs. 59%, p=0.44). The size of the ovulatory follicle at the time of CIDR removal and at AI was larger in the long-progesterone groups than in short-progesterone groups (P=0.01). The size of ovulatory follicle at the time of CIDR removal was similar between heifers and cows (P=0.48) but was larger in cows than heifers at the time of AI (P=0.001). To conclude, results did not support the stated hypothesis and, hence, do not support the concept that oocyte competence is different in estrous cycles with 2- vs. 3-follicular waves.

Introduction

- The duration of the ovulatory wave in 2-wave cycles is about 9 days, and only 6 days in 3-wave cycles.
- Ovulatory waves differ between 2 and 3-wave cycles in the duration of high-progesterone period (i.e., 6 days vs. 3 days respectively) while the progesterone free period after luteolysis is similar (3 days).
- Some reports suggest that fertility is higher in 3-wave cycles than fertility in 2-wave cycles; Other reports suggest no difference in fertility rate between 3-wave and 2-wave cycles.

Objective and Hypothesis

Objective

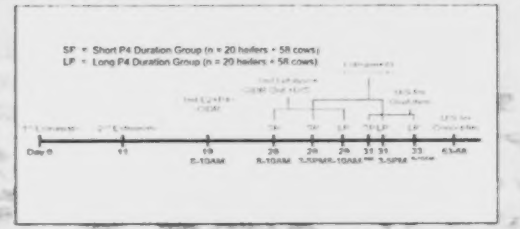
- To determine the effect of the duration of progesterone influence during the growing phase of the ovulatory wave on fertility (pregnancy rate).

Hypothesis

- A short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles).

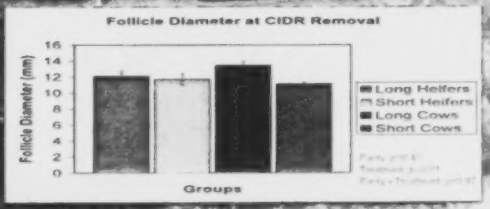
Procedures

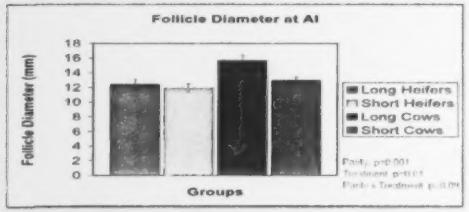
- Heifers (n=40) and cows (n=116) at random stages of estrous were treated with prostaglandin (PGF) analog (estrumate 2ml i/m) injections at 11-day intervals to synchronize estrus and ovulation.
- On Day 3-5 of the new cycle (8 days after 2nd PFG injection) all animals received estradiol-progesterone injection (2.5mg estradiol 17beta+50mg Progesterone in 1ml No-name Canola oil i/m) and an intravaginal CIDR-B device to synchronize emergence of a new follicular wave 4 days later. The CIDR was expected to raise plasma progesterone concentrations to mid-luteal values, analogous to the time of emergence of the ovulatory wave in 2- and 3- wave cycles.
- The short duration group (n=20 heifers and 58 cows) were given PGF (5ml Lutalyse i/m) 3 days after emergence of the new follicular wave (7 days after E2+P4 injection).
- The long duration group (n=20 heifers and 58 cows) were given PGF (5ml Lutalyse i/m) 6 days after emergence of the new follicular wave (10 days after E2+P4 injection).
- CIDR device was removed at the time of PGF injection. Both groups were given LH (2.5ml Lutropin i/m, i.e., half of standard dose) 55hr after CIDR removal. Animals were insemination (AI) at this time.
- Follicle and CL size were measured by transrectal ultrasonography at the time of PGF injection, artificial insemination and 36 hours post AI. Pregnancy diagnosis was performed by ultrasonography 30-35 days post AI.



Results

Pregnancy Rate	Short Progesterone	Long Progesterone	P-Value (Chi-Sq.)
Cows	38/58 (66%)	34/58 (59%)	0.44
Heifers	14/20 (70%)	9/20 (45%)	0.1
Total	52/78 (67%)	43/78 (55%)	0.11





Interpretation

- Pregnancy rates between cattle in long progesterone groups (analogous to 2-wave cycles) and those in short progesterone group (analogous to 3-wave cycles) show no statistical difference (p>0.11).
- Parity (cow vs. heifers) did not affect the pregnancy rate.
- Diameter of the ovulatory follicle was larger than 12mm in all groups.
- Ovulatory follicle of cows and Heifers in the long progesterone exposure groups had larger diameter (p=0.01) at CIDR removal and at the time of artificial insemination than that of short progesterone groups. This finding was expected as the life span of the dominant follicle was longer by 3 days in long progesterone group.
- Cows in the long progesterone group had a larger ovulatory follicle at the time of artificial insemination than the heifers in long progesterone group.
- For practical reasons, length of the induced ovulatory wave (emergence to ovulation) can be varied between 8 to 11 days.

Conclusions

- The hypothesis that short period of progesterone exposure during the growing and early-static phase of the dominant follicle is associated with higher fertility than a long period of progesterone exposure was *Not Supported*.
- There is no difference in the oocyte competence in estrous cycles with 2- vs. 3-follicular waves.
- There was no effect of parity (cows vs. heifers) on fertility. Fixed-time insemination was equally effective in both cows and heifers.

This project was supported by research grants from Saskatchewan Agriculture Development Fund, NSERC and Ferring Inc. We acknowledge the in-kind contributions from Veterinary Pharmaceutical Companies (Bayer Inc., Pfizer and Intervet Canada).

Proceedings of the 39th Annual Meeting of the Society for the Study of Reproduction (2006)

OOCYTE COMPETENCE UNDER DIFFERENT PROGESTATIONAL ENVIRONMENTS

Jaiswal RS, Singh J, Nagra HS, Grafton T, Ratto MH, Malhi PS and Adams GP

Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon S7N 5B4 Canada.

Prolonged treatment with progestagens for estrus synchronization results in poor fertility. The objectives of this study were to compare the developmental capacity of oocytes that develop 1) under low- versus high-progesterone concentrations, and 2) from persistent dominant follicles. Hereford crossbred heifers with a functional corpus luteum (CL) were assigned randomly to three groups (n=10/group) intended to provide low-, prolonged-low- and high- circulating progesterone (P4). Follicular wave emergence was synchronized among heifers by follicular ablation, and low-P4 implants (once-used CIDR) were placed intravaginally. Heifers in low-P4 and prolonged-low-P4 groups were treated with 500μg PGF2α analog 12h apart on the day of ablation while heifers in high-P4 group were allowed to retain their CL. Beginning 24h post-ablation, heifers in all groups were treated i.m. with 200mg FSH divided bid over 4 days (Day 0=first FSH treatment). Heifers in high-P4 group were given PGF2α on Day 3. The CIDR devices were removed from heifers in low-P4 and high-P4 groups on Day 3, and from prolonged-low-P4 group on Day 8. All heifers were treated i.m. with 25mg LH 24h after CIDR removal. Heifers were artificially inseminated at 24h and 36h post-LH treatment and were sent to slaughter 3 days after the last insemination. The embryos/ova were flushed from the uterine tubes. Ovarian ultrasonography was conducted on Days -1, 4, and 24h prior to slaughter. Data were analyzed using one-way ANOVA and Chi-squared tests. Plasma LH pulse frequency (36h before CIDR removal) was lowest and Day 3 P4 concentration was highest in high-P4 group ($P<0.01$). The number of follicles ≥12mm was highest ($P=0.02$) and the number of follicles 9 to11 mm tended to be higher ($P=0.07$) in prolonged-low-P4 group. No ovulations were detected in prolonged-low-P4 group. The embryo/ovum recovery rate was higher ($P<0.01$) in high-P4 (139/204, 68%) versus low-P4 (109/218, 50%) group, but the proportion of fertilized ova was higher ($P<0.01$) in low-P4 (94%) versus high-P4 group (73%). It was concluded that in FSH treated heifers, oocytes that developed under a high-progesterone milieu were less capable of developing into embryos, whereas oocytes that developed under a prolonged low- progesterone milieu failed to be ovulated.

Supported by Saskatchewan ADF, Westgen, Pfizer, Bioniche, Schering-Plough Animal Health and NSERC.

Final Report – ADF Project # 20050731

OPTIMIZING FERTILITY AND OOCYTE QUALITY AFTER ARTIFICIAL INSEMINATION IN BEEF CATTLE

Manuscripts Submitted for Publication

with Saskatchewan ADF Funding Support

Duration of progesterone exposure during development of the ovulatory follicle does not affect pregnancy rate in cattle

Dias F.C.F.[1], Mapletoft R.J.[2], Kastelic J.[3], Adams G.P.[1], Colazo M.[4], Stover B.C.[1], Dochi O.[5], Singh J.[1*]

[1]Department of Veterinary Biomedical Sciences and [2]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada
[3, 4]Agriculture and Agri-Food Canada, Lethbridge Research Centre, Lethbridge, Canada.
[5] Department of Dairy Science, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan,

*Corresponding author

Correspondence Address:

Dr. Jaswant Singh, Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, S7N 5B4, Canada.
Phone: 1-306-966-7410
Fax: 1-606-966-7405
Email: jaswant.singh@usask.ca

DRAFT Confidential

Abstract

The objective was to determine the effect of duration of progesterone exposure on the ovulatory wave on pregnancy rates in beef cattle. We tested the hypothesis thata short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles).Three to 5 d after ovulation, beef heifers (n=172) and suckled beef cows (n=193) received an intravaginal insert containing 1.9 g progesterone (CIDR) and 2.5 mg estradiol-17β + 50 mg progesterone im to induce a new follicular wave. Cattle were allocated to short- or long-progesterone exposure groups (for 3 and 6 d after wave emergence, respectively), analogous to the ovulatory wave of 3- and 2-wave estrous cycles., At the end of progesterone treatment, prostaglandin $F_{2\alpha}$ was administered and CIDR inserts were removed. Forty-eight hours later, all cattle were given 12.5 mg pLH, and concurrently artificially inseminated with frozen-thawed semen. Diameter of the two largest follicles and the CL were measured by transrectal ultrasonography at CIDR removal, insemination, and 36h after insemination. Ultrasonographic pregnancy diagnosis was done 38 and 65 d after AI. There was no difference in pregnancy rates following short- versus long-duration progesterone exposure in heifers (53 vs 47%, P=0.44) or cows (63 vs 58%, P=0.51). However, the diameter of the ovulatory follicle at CIDR removal and AI was smaller in short- than in long-duration groups (P<0.02), and larger in cows than in heifers (P<0.006). In conclusion, although long-duration progesterone exposure during growth of the ovulatory follicle resulted in a larger diameter on the last examination before ovulation, pregnancy rate was not significantly different than in the short-progesterone exposure group.

Key words: Cattle; Fertility; Follicular waves; Heifer; Ovarian follicle; Ovary; Progesterone; Postpartum cows.

1. Introduction

Follicles develop in a wave-like pattern (1,12,21), with most cattle having either 2 or 3 follicular waves during each estrous cycle (21). However, the preponderance and repeatability of 2- or 3-wave cycles remain unclear (16). The duration of exposure of ovulatory follicles to high-progesterone concentrations is substantially different between 2- and 3-wave cycles. The duration of the ovulatory wave in 2-wave cycles is approximately 9 d, whereas in 3-wave cycles, the ovulatory wave lasts only 6 d (12). Although progesterone concentrations are consistently elevated when the ovulatory wave emerges, the dominant follicle grows in a high-progesterone environment for approximately 6 d in 2-wave cycles, but only 3 d in 3-wave cycles. Thereafter, progesterone concentrations are low (for both 2- and 3-wave cycles) for approximately 3 d after luteolysis, culminating in ovulation of the dominant follicle (12,13). Hence, the interval from wave emergence to ovulation and the duration of growth in a high-progesterone milieu are longer in 2-wave than 3-wave cycles (19). It has been suggested that follicles which grow for a longer interval in a high-progesterone environment (analogous to 2-wave cycles) may produce an aged oocyte (19). Although fertility was higher in 3-wave than in 2-wave cycles in one report (19), others have failed to detect a difference (4), suggesting that further study is required.

The objective was to determine the effect of the duration of progesterone exposure, during the growing phase of the ovulatory follicle on pregnancy rate. We hypothesized that a short period of progesterone exposure during the growing and early-static phase of the dominant follicle (analogous to the ovulatory wave of 3-wave cycles) is associated with higher fertility than a long period of progesterone exposure (analogous to the ovulatory wave of 2-wave cycles).

2. Materials and methods

2.1. Cattle and treatments

The experiment was conducted between May and September 2006 with pubertal cross-bred beef heifers (n=172) and suckled, post-partum beef cows (n=193), maintained in outdoor corrals at three locations: University of Saskatchewan Goodale Research Farm, University of Alberta Research Farm, and Brandon Research Centre. All procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee or the University of Alberta Animal Welfare Committee.

Animals at random stages of the estrous cycle were given a prostaglandin $F_{2\alpha}$ analog, 500 µg of cloprostenol im (Estrumate; Schering-Plough Animal Health, Pointe-Claire, QC, Canada), on two occasions, 11 d apart, to synchronize estrus and ovulation (ovulation = Day 0). Eight days after the second cloprostenol treatment (Days 3 to 5), all cattle received an intravaginal device containing 1.9 g of progesterone (CIDR , Pfizer Canada Inc., Montreal, QC, Canada) and they were injected im with 1 ml of canola oil containing 2.5 mg estradiol-17β + 50 mg progesterone (E_2+ P_4; Sigma Chemical Co., St. Louis, MO, USA); a new ovarian follicular wave was expected to emerge 4 d later (5,6). The CIDR and functional CL were expected to raise plasma progesterone concentrations to mid-luteal values, analogous to the time of emergence of the ovulatory wave in both 2- and 3-wave cycles.

DRAFT (Confidential)

Heifers and cows were randomly allocated into two groups. The short duration of progesterone exposure (short-progesterone) group was given 25 mg of prostaglandin $F_2\alpha$ im ($PGF_2\alpha$; Lutalyse, Pfizer Canada Inc.) 3 d after expected emergence of the new follicular wave (i.e., 7 d after E_2+P_4). The long duration exposure of progesterone (long-progesterone) group was given $PGF_2\alpha$ 6 d after expected emergence of the new follicular wave (i.e., 10 d after treatment with E_2+P_4). The CIDR devices were removed concurrent with $PGF_2\alpha$ treatment. Forty-eight hours later, all cattle received 12.5 mg porcine luteinizing hormone im (Lutropin-V; Bioniche Animal Health, Belleville, ON, Canada) and were concurrently inseminated with frozen-thawed semen.

2.2. Ultrasonographic examination

Transrectal ultrasonography was performed using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan); diameter of the two largest follicles and all visible corpora lutea were measured at CIDR removal at all three locations. Follicle and CL diameter were also measured at the time of AI and 36 h after AI to confirm ovulation in a subset of animals at the University of Saskatchewan Goodale Research Farm (n=87 heifers and n=57 cows). Ultrasonographic pregnancy diagnosis was performed at all three locations 38 d after AI. To determine pregnancy loss, an additional ultrasonographic examination was conducted in cows and heifers from the University of Saskatchewan Goodale Research Farm 65 d after AI.

2.3. Blood sampling and radioimmunoassay

Blood samples were collected from the coccygeal vessels into 10 ml heparinized tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) at CIDR removal and at AI to determine plasma concentrations of LH and progesterone. Blood samples were centrifuged (1500 x g for 15 min), and plasma was stored at -20 °C until analyzed.

Plasma LH concentrations were measured in duplicate samples in a single assay, using a validated, double-antibody radioimmunoassay (7). The range of the standard curve was 0.06 to 8.0 ng/ml and the intra-assay coefficients of variation were 6% for the low-reference serum (1.02 ng/ml) and 7% for the high-reference serum (2.18 ng/ml).

Plasma progesterone concentrations were measured in a single assay using commercial radioimmunoassay kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40 ng/ml. The intra-assay coefficients of variation were 6% for low-reference serum (1.84 ng/ml), 6% for medium-reference serum (3.5 ng/ml), and 6% for high-reference serum (17.06 ng/ml).

2.4. Statistical analyses

Eliminate one or two sentence paragraphs; must combine them into longer paragraphs.

Statistical analyses were performed using the Statistical Analysis System software package (SAS Version 9.0; SAS Institute Inc., Cary, NC, USA). The effects of treatment (long and short-progesterone) and parity were compared for diameters of the largest follicle and CL and for plasma concentrations of LH and progesterone, by 2-way ANOVA using Proc MIXED. Normality of residuals was tested with a Shapiro-Wilk test ($P>0.05$ was considered a normal distribution). Values are expressed as mean ± SEM.

Pregnancy data were analyzed by 2-way factorial with randomized complete block design (Fixed factors: duration of progesterone exposure, parity, duration of progesterone exposure*parity interaction; Random factor: location). Data collected at one location (follicle diameter at AI) were

Confidential Draft

analyzed by 2x2 factorial design (length of progesterone exposure, parity and length of progesterone exposure *parity interaction). Tukey's post-hoc test for multiple comparisons was used if main effects or their interaction were significant ($P \leq 0.05$).

Dominant follicle growth during the pre-ovulatory period was determined by the difference in diameter of dominant follicle between CIDR removal and AI, and was also analyzed by 2-way ANOVA.

For plasma LH data, concentrations three times higher than the standard deviation of the mean LH concentration at the time of AI (i.e., cattle with an endogenous LH peak) were excluded from the analysis.

Animals which had CL <15 mm in diameter and plasma progesterone <1.5 ng/ml at CIDR removal were considered outliers; two analyses of pregnancy rates were performed (excluding and including this group of cattle).

All dichotomous variables (pregnancy rates at 38 days post-AI; pregnancy loss; diameter CL < 15 mm, and progesterone concentrations <1.5 ng/ml; ovulation rate; and detection of early ovulation) were analyzed using logistic regression with the Gen Mod procedure. [This is good information, but it appears far too late in the description of the analyses; it should be incorporated earlier.]

3. Results

3.1. Dominant follicle size and growth

The diameter of the ovulatory follicle at CIDR removal and AI were larger in long-progesterone than in short-progesterone cows, but did not differ significantly in heifers (Fig. 3.1). The diameter of the ovulatory follicle at CIDR removal and AI were larger in long-progesterone cows than long-progesterone heifers (Fig. 3.1). Dominant follicle growth during the pre-ovulatory period did not differ between progesterone treatment groups (P=0.61), but was higher in cows than heifers (P=0.0005).

Fig. 3.1. Mean (± SEM) follicle diameter at CIDR removal and AI in cows and heifers exposed to long- or short-duration progesterone environments during development of the ovulatory wave.

[a,b]Values without a common superscript were different (P<0.05).

3.2. Diameter of CL and plasma progesterone concentration

Diameter of the CL at CIDR removal was greater in cows in the short-progesterone group than in cows and heifers in the long-progesterone groups, but not in heifers in the short-progesterone group (Fig. 3.2). There was no difference in CL diameter at CIDR removal between heifers in the short and long-progesterone groups (Fig. 3.2).

Fig. 3.2 Mean (± SEM) diameter of the CL at CIDR removal and at AI in heifers and/or cows exposed to long- or short-duration progesterone environments during development of the ovulatory wave.

[a,b]Values without a common superscript were different (P<0.05).

DRAFT Confidential

[The following paragraphs are really tedious to read. I suggest you incorporate these data into a table, and then VERY BRIEFLY highlight them in the text, without repeating a lot of detail I suggest that your columns should be as follows: End point, 4 groups, Main effects, Interaction

Plasma progesterone concentrations at CIDR removal were not different between groups (4.8 ± 0.8 in short-progesterone heifers, 4.9 ± 0.8 in long-progesterone heifers, 4.3 ± 0.6 in short-progesterone cows, and 3.2 ± 0.6 in long-progesterone cows; P=0.3, 0.3 and 0.2 for group, location, and their interaction, respectively). However, progesterone concentrations at CIDR removal were higher in heifers (4.9 ± 0.7 ng/mL) than in cows (3.8 ± 0.6 ng/mL; P=0.02). Progesterone concentrations at AI were less than <0.5 ng/mL in all groups, and did not differ (0.4 ± 0.2 ng/mL in short-progesterone heifers, 0.3 ± 0.2 ng/mL in long-progesterone heifers, 0.4 ± 0.1 ng/mL in short-progesterone cows and 0.3 ± 0.1 ng/mL in long-progesterone cows; P=0.29, 0.64, 0.7, and 0.3 for group, parity, interaction, and location).

A greater proportion of cows in the long-progesterone group had a CL <15 mm in diameter and plasma progesterone concentrations <1.5 ng/mL at CIDR removal (21.4%; 21/98) than cows in the short-progesterone group (8.4%; 8/95), heifers in the long-progesterone group(3.5%; 3/86), and heifers in the short-progesterone group (1.2%; 1/86; P=0.005, 0.0006, and 0.9 for group, parity, and their interaction, respectively).

3.3. Plasma LH concentrations and ovulation rates

Plasma LH concentrations at CIDR removal did not differ by group, parity or interaction. However, LH concentrations at AI were higher in heifers in the long-progesterone group than in the other groups (Fig. 3.3).

Fig. 3.3 Mean (± SEM) plasma concentrations of LH at CIDR removal and at AI in heifers and cows exposed to long- or short-duration progesterone environments during development of the ovulatory wave. [a,b]Values without a common superscript were different (P<0.05)

There were no significant differences between groups, parity or interaction, for the proportion of cattle that ovulated by 36 h after pLH treatment (P=0.89, 0.23 and 0.6, respectively). Ovulation rates were 87% (41/47) in the long-progesterone group (3 cows and 4 heifers did not ovulate by 36 h after AI), Ovulation rates were 88.9% (16/18) in long-progesterone heifers, 84% (16/19) in short-progesterone heifers, 89.6% (26/29) in long-progesterone cows and 89.3% (25/28) in short-progesterone cows. For early ovulations (i.e., ovulations between CIDR removal and AI, P=0.32, 0.19, 0.9, respectively), there were no difference due to group, parity, or their interaction. One cow ovulated before AI in the short-progesterone group and 3 heifers ovulated before AI in the long-progesterone group.

3.4. Pregnancy rates

There were no differences in pregnancy rates between treatment groups (P=0.32, Table 3.1), parities (P=0.6, Table 3.1), or interactions (P=0.9). Location did not affect pregnancy rates at 38 d (P=0.6), and there was no difference in pregnancy loss between groups, parities or interactions (P=0.7; 0.85, 0.8) at the University of Saskatchewan Research farm. The overall incidence of pregnancy loss was 7.4% (1/19 in short-progesterone heifers, 1/19 in long-progesterone heifers, 3/28 in short-progesterone cows, and 1/29 in long-progesterone cows).

DRAFT Confidential

When pregnancy rates at 38 d were analyzed, excluding cattle with <1.5 ng/ml of progesterone and CL diameter <15 mm at CIDR removal, there were no differences due to group (P=0.5), parity (P=0.3), interaction (P=0.3), or location (P=0.9).

Table 3.1 Pregnancy rates in cows and heifers exposed to a short or long-progesterone environment during development of the ovulatory wave.

Pregnancy rate	Short-progesterone	Long-progesterone
Cows	59/94 (63%)	58/99 (58%)
Heifers	45/85 (53%)	41/87 (47%)
Total	104/179 (58%)	99/186 (53%)

4. Discussion

The duration of progesterone exposure during ovulatory wave development did not significantly affect pregnancy rates. In that regard, the dominant follicle of both short-progesterone (analogous to the ovulatory wave of 3-wave cycles) and long-progesterone groups (analogous to the ovulatory wave of 2-wave cycles) ovulated, resulting in acceptable pregnancy rates after fixed-time AI, with a low incidence of subsequent pregnancy loss. Therefore, the hypothesis that short-duration progesterone exposure during the growing and early-static phase of the ovulatory follicle is associated with higher fertility than long-duration exposure was not supported.

Although diameter of the ovulatory follicle at CIDR removal and AI were larger in cows in the long-progesterone groups, pregnancy rates did not differ between groups, or between parities. Thus, follicles from heifers and from short-progesterone cows were smaller, but they apparently had the same potential for ovulation, fertilization, and maintenance of pregnancy. Cows have been reported to have larger ovulatory follicles than heifers (17,22), and cattle from the long-progesterone group had a follicular growing phase 3 d longer than those in the short-progesterone groups. Ovulatory follicles <11 mm in diameter at AI yielded reduced pregnancy rates when ovulation was induced with GnRH (15). In that regard, larger follicles contained more granulosa cells, which after luteinization resulted in a larger CL that produced more progesterone (14,15). However, in the present study, it was noteworthy that preovulatory follicles from all cattle in all groups exceeded 11 mm at AI; this may account for our inability to detect an effect of follicle diameter at AI on pregnancy rates.

The duration of follicle dominance has also been reported to affect fertility (2,3). In beef heifers, 4 d of follicle dominance resulted in the highest pregnancy rates; as the duration of follicular dominance increased, fertility decreased gradually until 9 d of dominance and thereafter, fertility declined rapidly (3). In that regard, prolonged follicular dominance results in formation of a persistent dominant follicle, with an aged oocyte and compromised developmental competence (3). When LH release was delayed in a superstimulation protocol by prolonging high progesterone concentrations to allow smaller follicles to fully mature, the number of ova/embryos retrieved was increased, but embryo production was either not affected or it was decreased (11). Conversely, it has been suggested that prolonged maintenance of the oocyte in the follicular environment could be beneficial to developmental competence, since it could accumulate more maternal mRNA, which is important for early embryonic development (11,20). However, in the present study,

DRAFT (Confidential)

shortening or extending the follicular growing phase (and dominance) with exogenous progesterone did not significantly affect pregnancy rates.

Cows in the long-progesterone group had lower progesterone concentrations and smaller CL diameters at CIDR removal, suggesting that some of these cattle were undergoing luteolysis. This may have been an effect of postpartum interval, since short cycles (approximately 8 d) are common in postpartum suckled beef cows (18). To avoid the confounding effect of short cycles on the results, cattle with progesterone concentrations <1.5 ng/mL at CIDR removal were removed from analyses of pregnancy rates. However, pregnancy rates still did not differ among groups. Cattle from all groups responded to PGF treatment and progesterone concentrations were baseline at the time of AI.

In the present study, plasma LH concentrations at CIDR removal were not significantly affected by group or parity. However, LH concentrations at AI in heifers in the long-progesterone group were significantly higher than in the other groups, suggesting that an LH surge was already underway. In other studies in heifers (9) and cows (10) in which treatments were similar to the short-progesterone group in this study, estrous behavior was detected 52 h after CIDR removal . Cattle usually initiate estrus approximately 2 h after their LH peak, therefore cattle from that study might have had an LH peak 50 h after CIDR removal. Similarly, when norgestomet ear implants were left in place for 8 d after wave emergence, beef heifers showed estrous behavior 39 h after ear implant removal (an LH peak approximately 37 h after norgestomet removal) (3). Therefore, a longer duration of progesterone/progestin exposure during growing phase of ovulatory follicle can result in a short interval to LH release in heifers.

Most pregnancy loss occurs in the first 45 d of pregnancy (2). The incidence of pregnancy loss between 38 and 65 days of pregnancy in the current study was 7.4%, similar to previous reports (8), with no significant difference between groups or parities.

In summary, although there were differences in follicular, CL and hormone profiles when the duration of the ovulatory wave was extended by 3 d with exogenous progesterone, there was no apparent affect on oocyte competence, manifested as a lack of significant difference in pregnancy rates.

Acknowledgements

The project was supported by research grants from Saskatchewan Agriculture Development Fund, Westgen Inc. and the Natural Sciences and Engineering Research Council of Canada. The authors thank Pfizer Animal Health for CIDR inserts and Schering-Plough Animal Health for Estrumate. The authors thank personnel from the Goodale farm for providing cattle and technical assistance.

DRAFT Confidential

REFERENCES

1. Adams GP, Nasser LF, Bo GA, Garcia A, Del-Campo MR, Mapletoft RJ. Superovulatory response of ovarian follicles of wave 1 versus wave 2 in heifers. Theriogenology 1994;42: 1103-1113.
2. Alnimer MA, Husein MQ. The effect of progesterone and oestradiol benzoate on fertility of artificially inseminated repeat-breeder dairy cows during summer. Reprod Domest Anim 2007;42: 363-369.
3. Austin EJ, Mihm M, Ryan MP, Williams DH, Roche JF. Effect of duration of dominance of the ovulatory follicle on onset of estrus and fertility in heifers. J anim sci 1999;v. 77: 2219-2226.
4. Bleach EC, Glencross RG, Knight PG. Association between ovarian follicle development and pregnancy rates in dairy cows undergoing spontaneous oestrous cycles. Reproduction 2004;127: 621-629.
5. Bo GA, Bergfelt DR, Brogliatti GM, Pierson RA, Adams GP, Mapletoft RJ. Local versus systemic effects of exogenous estradiol-17 beta on ovarian follicular dynamics in heifers with progestogen implants. Anim Reprod Sci 2000;59: 141-157.
6. Colazo MG, Martinez MF, Small JA, Kastelic JP, Bumley CA, Ward DR, Mapletoft RJ. Effect of estradiol valerate on ovarian follicle dynamics and superovulatory response in progestin-treated cattle. Theriogenology 2005;63: 1454-1468.
7. Evans AC, Adams GP, Rawlings NC. Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. J Reprod Fertil 1994;100: 187-194.
8. Galvao KN, Santos JE, Cerri RL, Chebel RC, Rutigliano HM, Bruno RG, Bicalho RC. Evaluation of methods of resynchronization for insemination in cows of unknown pregnancy status. J Dairy Sci 2007;90: 4240-4252.
9. Martinez MF, Adams GP, Kastelic JP, Bergfel DR, Mapletoft RJ. Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. Theriogenology 2000;54: 757-769.
10. Martinez MF, Kastelic JP, Colazo MG, Mapletoft RJ. Effects of estradiol on gonadotrophin release, estrus and ovulation in CIDR-treated beef cattle. Domest Anim Endocrinol 2007;33: 77-90.
11. Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003;59: 651-674.
12. Mihm M, Crowe MA, Knight PG, Austin EJ. Follicle wave growth in cattle. Reprod Domest Anim 2002;37: 191-200.
13. Nosair WM. Ovarian follicular activity and hormonal profile during estrous cycle in cows: the development of 2 versus 3 waves. Reprod Biol Endocrinol 2003;1: 50.
14. Oussaid B, Lonergan P, Khatir H, Guler A, Monniaux D, Touze JL, Beckers JF, Cognie Y, Mermillod P. Effect of GnRH antagonist-induced prolonged follicular phase on follicular atresia and oocyte developmental competence in vitro in superovulated heifers. J Reprod Fertil 2000;118: 137-144.
15. Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, Roberts AJ, Geary TW. Relationship between follicle size at insemination and pregnancy success. Proc Natl Acad Sci U S A 2005;102: 5268-5273.
16. Rajesh SJ. Regulation of follicular wave pattern in cattle, Department of Veterinary Biomedical Science. Saskatoon: University of Saskatchewan, 2007;169.

DRAFT Confidential

17. Sartori R, Rosa GJ, Wiltbank MC. Ovarian structures and circulating steroids in heifers and lactating cows in summer and lactating and dry cows in winter. J Dairy Sci 2002;85: 2813-2822.
18. Taponen J, Katila T, Rodriguez-Martinez H. Induction of ovulation with gonadotropin-releasing hormone during proestrus in cattle: influence on subsequent follicular growth and luteal function. Anim Reprod Sci 1999;55: 91-105.
19. Townson DH, Tsang PC, Butler WR, Frajblat M, Griel LC, Jr., Johnson CJ, Milvae RA, Niksic GM, Pate JL. Relationship of fertility to ovarian follicular waves before breeding in dairy cows. J Anim Sci 2002;80: 1053-1058.
20. Vassena R, Mapletoft RJ, Allodi S, Singh J, Adams GP. Morphology and developmental competence of bovine oocytes relative to follicular status. Theriogenology 2003;60: 923-932.
21. Vinoles C, Meikle A, Forsberg M, Rubianes E. The effect of subluteal levels of exogenous progesterone on follicular dynamics and endocrine patterns during early luteal phase of the ewe. Theriogenology 1999;51: 1351-1361.
22. Wolfenson D, Inbar G, Roth Z, Kaim M, Bloch A, Braw-Tal R. Follicular dynamics and concentrations of steroids and gonadotropins in lactating cows and nulliparous heifers. Theriogenology 2004;62: 1042-1055.

DRAFT (Confidential)

Effect of length of progesterone and growing phase of the ovulatory follicles on oocyte competence after superstimulatory treatment

Dias F.C.F.[1], Costa E.[1], Adams G. P.[1], Mapletoft R. J.[2], Dochi O.[3], Singh J.[1]

[1]Department of Veterinary Biomedical Sciences and
[2]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 5B4
[3] Rakuno Gakuen University, Ebetsu 069-8501, Japan

Address for Correspondence:

Dr. Jaswant Singh, Department of Veterinary Biomedical Sciences, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, S7N 5B4, Canada
Phone: 1-306-966-7410
Fax: 1-306-966-7405
Email: jaswant.singh@usask.ca

DRAFT (Confidential)

Abstract

The objective was to determine the effect of duration of progesterone exposure, during the growing phase of ovulatory follicles, on oocyte competence in superstimulated cows. We tested the hypotheses that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth, but is not affected by FSH starvation at the end of superstimulatory treatment. Forty cross-bred beef cows (weight, 515 to 795 kg) were used. Transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter was done 5 to 8 days after ovulation, and a progesterone-releasing device (CIDR) was placed intravaginally. Cows were allocated randomly to three groups; those in the short P_4 and FSH starvation groups received 8 injections of FSH (each was equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals, whereas the long P_4 group received 14 injections of FSH. In all groups, FSH treatment started on the day of wave emergence (Day 0). Cows in the short P_4 group were given 25 mg of PGF twice, (12 hours apart), on Day 3, whereas cows in the 2 other groups received two injections of PGF on Day 5. In all cows, the CIDR were removed at the time of second PGF treatment; 25 mg pLH was given im 24 hours after CIDR removal and cows were inseminated 24 and 36 hours later. Reproductive tracts were collected at slaughter, 4 days after insemination and ova/embryos were evaluated and cultured for 10 days to determine developmental competence. Data from cows that failed to ovulate (6 of 13 and 1 of 13 in the FSH starvation and long P_4 groups, respectively), were excluded from the analyses. When compared to the short P_4 or long P_4 groups, the FSH starvation group had fewer CL (i.e.

ovulations; mean±SEM, 11.6±2.2, 15.4±3.0, and 2.5±1.2, respectively; P=0.001), and fewer ova or embryos (5.9±1.3, 6.7±1.5, and 0.9±0.6, respectively; P<0.05) at slaughter. However, the proportion of Grades 1 and 2 embryos 4 days after insemination was higher in the short P_4 and FSH starvation groups than in the long progesterone group (60 of 69, 9 of 11, and 31 of 82, respectively; P<0.001), with no significant difference in embryo quality between long and short P_4 groups 7, 9, and 10 days after AI (there were too few embryos in the FSH starvation group to evaluate). In conclusion, the hypothesis that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth was not supported, whereas the hypothesis that oocyte competence is not affected by FSH starvation at the end of superstimulatory treatment was not adequately tested. Notwithstanding, FSH starvation substantially reduced the ability of superstimulated follicles to ovulate.

Introduction

Ovarian follicles in cattle develop in a wave-like pattern, with 2 or 3 follicular waves in each estrous cycle [12, 123]. The major difference between 2- and 3-wave cycles is the length of growing phase of the ovulatory follicle [5]; the duration of the ovulatory wave is 3 days longer in 2-wave cycles (6 versus 9 days [5]). Therefore, the ovulatory oocyte is 3 days older (relative to wave emergence) in 2-wave cycles [14].

In cattle with 2 waves, the ovulatory follicle is exposed to high progesterone concentrations (from a functional CL) for 3 days longer than the ovulatory follicle in cattle with 3 waves [14]. Progesterone suppresses GnRH and LH in a dose-specific manner [12]. A pre-ovulatory peak of LH is required for the oocyte to reactivate nuclear maturation and achieve developmental competence; however, the LH peak could be delayed 3 days in females with 2-wave cycles (relative to wave emergence) [92].

Although pregnancy rates in 2- versus 3-wave cycles have been compared [17, 115], the results were contradictory, and the effect of number of waves on fertility remains unclear. In addition, the effect of the duration of the growing phase of the ovulatory follicle in superstimulated cows has not been reported. Jaiswal and co-workers (2006) [2, 6] studied oocyte competence after superstimulatory treatment under the influence of various progesterone concentrations. However, in both 2- and 3-wave cycles, circulating progesterone concentrations declined and remained subluteal for approximately 3 days prior to ovulation [14]. Therefore, the difference between 2- and 3-wave cycles is in the duration of progesterone exposure and not in progesterone concentrations [14].

For many years, gonadotrophin treatments have been used in multiple ovulation and embryo transfer programs [81, 111]; however, a major limitation is the extreme variability in the superovulatory response [26, 113]. On average, five to six transferable embryos are produced per donor [71, 72]. In one study, 30% of 2048 cows produced 70% of the embryos, whereas 24% cows failed to produce any embryos [71]. It has been postulated that one of the major determinants of the superstimulatory response is the number of follicles available at the beginning of a wave [9], and when FSH treatment is initiated in relation to wave emergence [79]. However, in superstimulated cows, it is not known whether a shorter duration of progesterone exposure during the growing phase of the ovulatory wave will increase the superovulatory response or embryo quality.

DRAFT Confidential

Superstimulatory response may also be affected by treatment protocol, total dose, batch, purity (i.e. LH content), and type of gonadotrophin [26]. In one study, increasing the interval from the end of gonadotrophin treatments to removal of a progesterone-releasing device resulted in a decreased ovulatory response [6]. However, this process, referred to as FSH starvation, was done in a prolonged, low-progesterone environment and it is unclear whether the loss of the capacity to ovulate was due to a lack of FSH, or to the formation of large, persistent follicles.

The objective of the present study was to determine the effect of duration of progesterone exposure, during the growing phase of ovulatory follicles, on oocyte competence in superstimulated cows. We tested the hypotheses that oocyte competence in superstimulated cows: 1) is improved by short-duration progesterone exposure during follicular growth; but 2) is not affected by FSH starvation at the end of a superstimulatory treatment protocol.

Materials and methods

Animals and treatments

The experiment was conducted at the University of Saskatchewan Goodale Research Farm (52°N and 106°W), during February and March, 2007. Forty cross-bred beef cows, weighing 515 to 795 kg, and maintained in outdoor pens, were used. All procedures were conducted in accordance with the guidelines of the Canadian Council on Animal Care and were approved by University of Saskatchewan Protocol Review Committee.

Cows at random stages of the estrous cycle were given 2 doses of prostaglandin $F_{2\alpha}$ im (500 µg of cloprostenol; Estrumate, Schering-Plough Animal Health, Pointe-Claire, PQ, Canada), at 14-day intervals, to synchronize estrus and ovulation. Transvaginal ultrasound-guided ablation of follicles ≥5 mm in diameter was done 5 to 8 days after ovulation, to synchronize emergence of a new follicular wave 1 day later [25]. An intravaginal, progesterone-releasing device, CIDR-B (Pfizer Canada Inc., QC, Canada) was placed in the vagina immediately after follicle ablation.

The cows were allocated randomly to three groups (Figure 4.1): short P_4 (n=14), FSH starvation (n=13), and long P_4 (n=13). Starting 1 day after follicle ablation, i.e. on the expected day of wave emergence (Day 0), cows in the short P_4 and FSH starvation groups received 8 im injections of FSH (Folltropin-V; Bioniche Animal Health, Belleville ON, Canada; each equivalent to 25 mg of NIH-FSH-P1) at 12-hour intervals over 4 days, whereas the long P_4 group received 14 im injections of FSH, over 7 days. Cows in the short P_4 group were given 2 im injections of 25 mg of prostaglandin-$F_2\alpha$ (Lutalyse®; Pfizer Canada Inc.) at 12-hour intervals on Day 3, whereas the FSH starvation and long P_4 groups received 2 injections of 25 mg im of prostaglandin-$F_2\alpha$ on Day 6. In all cows, the CIDR were removed at the time of the second $PGF_2\alpha$ treatment. Cows were given 25 mg pLH im (Lutropin-V, Bioniche Animal Health) 24 hours after CIDR removal, and artificially inseminated with frozen-thawed bull semen 24 and 36 hours later. Ovulations were monitored by transrectal ultrasonography, and a third AI was done 48 hours after LH treatment if ovulations were not completed at that time.

DRAFT Confidential

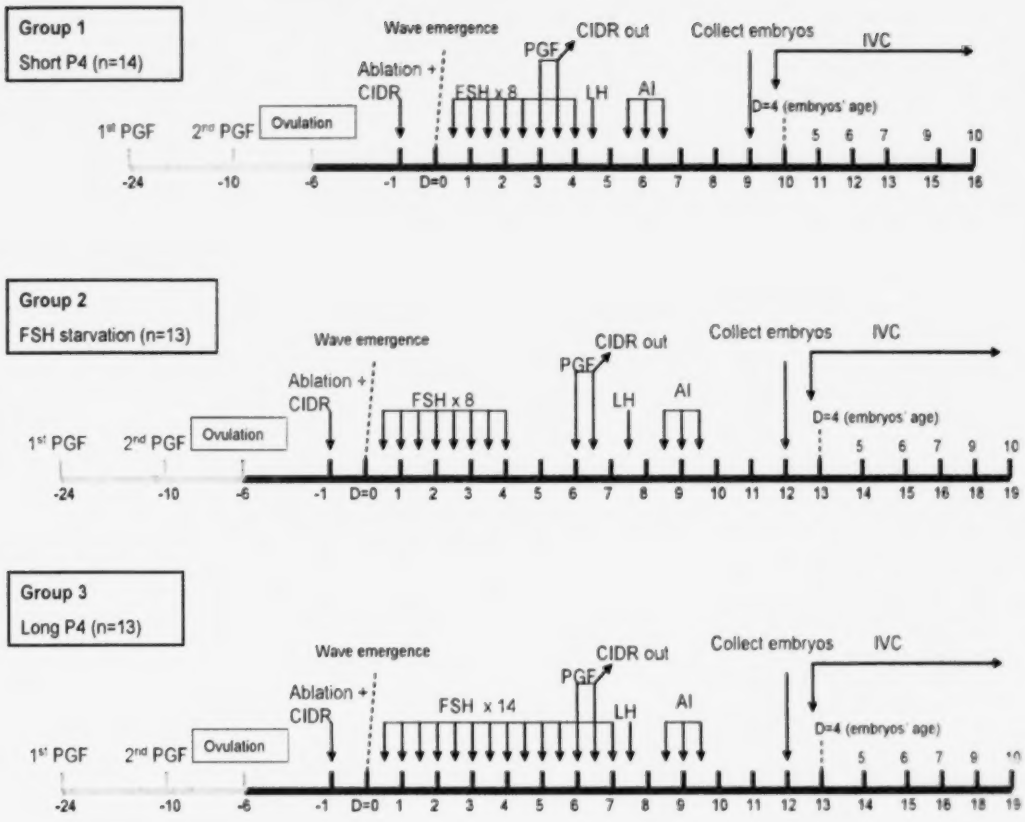


Figure 1 Experimental protocols used to test the effect of length of growing phase of the ovulatory follicles and FSH starvation on oocyte competence after superstimulatory treatment in cows. Five to 8 days after ovulation, follicles ≥5 mm were ablated and a CIDR was placed intravaginally. FSH treatment started at wave emergence (Day 0). Short P_4 and FSH starvation groups received 8 injections of FSH, whereas the long P_4 group received 14 injections of FSH. At Day 3, the short P_4 group received PGF, whereas the other groups received PGF on Day 6. CIDR were removed at the time of PGF, LH was given 24 hours after CIDR removal, cows were inseminated 24 and 36 hours later, and reproductive tracts were collected at slaughter, 4 days after AI.

DRAFT

Ultrasonographic examination

Transrectal ultrasongraphic imaging was performed using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan). To evaluate the number and size of ovulatory follicles, ultrasonography was done at the time of

8th FSH injection, and at CIDR removal (for the short P_4 group, those events were concurrent). Cows were also examined 12 hours after the second AI to detect ovulation; those with at least 2 follicles ≥7 mm in diameter were re-inseminated 48 hours after LH treatment.

The differences in number of follicles at CIDR removal and at 12 hours after the second AI were used to estimate the number of ovulations that occurred during this interval.

Embryo recovery

Cows were transported to an abattoir in Moose Jaw, SK, Canada, (approximately, 250 km from the location of the experiment) 3 days after the first AI. The cows were killed 4 days after AI; reproductive tracts were collected after slaughter, placed in polyethylene bags, kept at 30 °C, and transported to the laboratory at University of Saskatchewan within 3 hours after collection. The numbers of corpora lutea and persistent (unovulated) follicles were counted to estimate number of ovulations. Oviducts and the uterine horns were flushed separately to recover embryos. The oviduct was dissected free from the mesosalpinx and separated from the uterus, leaving approximately 1 cm of the tip of the uterine horn. An 18 gauge, 1.5 inch blunt needle was attached to the infundibulum with tissue forceps and 20 mL of collection medium was injected slowly; the fluid was collected from the uterine tip in a Petri dish for direct searching. The collection medium was comprised of Delbecco's phosphate buffer saline (dPBS, Invitrogen Corporation, catalog 14190-144, Burlington, ON, Canada) and 2% fetal calf serum. To flush the uterus, the base of each uterine horn was incised and a 60 ml catheter-tipped syringe filled with collection medium was placed in the incision. A large tissue clamp was placed just behind the syringe, preventing medium from flowing into the body of the uterus. Collection medium (60 ml for each uterine horn) was passed through the tip of each uterine horn and filtered through a 70 micron embryo filter (Emcon filters; Veterinary Concepts; Spring Valley, WI, USA). Filters were washed with collection medium and sprayed with 20 mL dPBS using a syringe and a 25 gauge needle (to wash embryos into two petri dishes) and 0.2 ml fetal calf serum was added to each of the two petri dishes. Petri dishes were labeled and ova/embryos were searched using a stereo-microscope at 10X magnification. Recovered ova/embryos were evaluated for stage and quality at 50X magnification using International Embryo Transfer Society (IETS) criteria [124]. All recovered embryo/ova were placed in drops of embryo culture medium covered with mineral oil. Embryo culture medium primarily contained NaCl, KCl, sodium pyruvate, $NaHCO_3$, BME essential amino acids, MEM nonessential amino acids, L-glutamic acid, bovine serum albumin and gentamicine (CR1aa + 5% fetal calf serum) [125]. From 1 to 8 embryos were cultured per drop, using 5 µl of medium per embryo. The petri dishes with embryo in culture medium were placed in an incubator with 5% CO_2, 5% O_2 and 90% N_2 at 38.5 ^{0}C. Cleavage was evaluated 4 days after AI (immediately after recovery from the reproductive tract), and following in vitro culture, blastocyst and hatching rates were evaluated 7, 9, and 10 days after the first AI (approximately 6, 8, and 9 days after fertilization).

Blood sampling and radioimmunoassays

DRAFT (confidential)

To estimate progesterone and LH concentrations, blood samples were collected (by venipuncture) from the caudal coccygeal vessels into 10 ml heparinized, evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA), concurrent with the first and sixth FSH treatments, first PGF, and pLH treatments. Blood samples were centrifuged at 1500 x g for 15 minutes, and plasma was separated and stored at -20 °C until assayed.

Plasma LH concentrations were measured in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) [105]. The range of the standard curve was 0.06 to 8.0 ng/ml. Intra-assay coefficients of variation were 3.4 and 5.6% for the low and high reference sera (means of 0.94 and 2.08 ng/ml, respectively).

Progesterone concentrations were measured in a single assay using solid-phase commercial kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/ml. The intra-assay coefficients of variation were 4.3, 3.3, and 3.4% for low, medium, and high reference sera (means, 1.79, 3.64, and 16.81 ng/ml, respectively).

Statistical analyses

Statistical analyses were performed using Statistical Analysis System software package (SAS Learning Edition 4.1; SAS Institute Inc., Cary, NC, USA). Numerically dependent variables (number of follicles at the 8th dose of FSH; number of follicles ≥7 mm at the 8th dose of FSH; number of follicles at CIDR removal; number of follicles ≥7 mm at CIDR removal; number of follicles 12 hours after second AI; number of follicles ≥7 mm at 12 hours after second AI; difference between number of follicles ≥7 mm at CIDR removal and at 12 hours after AI; and number of CL at slaughter) were analyzed by one-way ANOVA, using a general linear model procedure (GLM). Normality of residuals was tested basing on Shapiro-Wilk test (P values >0.05 were considered nonsignificant). Numbers of CL at time of slaughter were used to determine ovulation rates.

Progesterone and LH concentration were analyzed by repeated measures (MIXED procedures). Tukey's post-hoc tests for multiple comparisons were performed if main effects were significant ($P \leq 0.05$). The values are expressed as mean ± SEM. Proportional variables (ova:embryo ratio; proportion of total and different grades of morula on Day 4; proportion of early blastocyst, blastocyst or expanded blastocyst on Days 9 and 10; proportion of transferable embryos (≥ early blastocyst from Grades 1, 2, and 3) were compared using Chi-Square or Fisher's exact test. Cows that failed to ovulate were excluded from the analysis.

DRAFT Confidential

Results

Follicular dynamics

There were tendencies for differences among groups for the number of follicles present at the 8th FSH treatment; however, the only significant difference was more follicles

≥7 mm in diameter at CIDR removal in the long P_4 group compared to the FSH starvation group (Table 1). More ovulations occurred between CIDR removal and 12 hours after the second AI in the long P_4 group than in the FSH starvation group (P=0.02), whereas the short P_4 group did not differ from either (Table 1). Several cows in all three groups had at least 2 follicles ≥7 mm at 12 hours after second AI and they received a third insemination 48 hours after pLH administration (9/14, 13/13, and 4/14 in short P_4, FSH starvation, and long P_4 groups, respectively).

Table 1 The effect of duration of progesterone exposure and FSH starvation on the mean (± SEM) number of follicles during superstimulatory treatments in cows.

	Short P_4	FSH starvation	Long P_4	P value
No. follicles	n = 14	n = 13	n = 13	-
8th dose of FSH				
≥3 mm	18.6 ± 2.5	19.2 ± 1.8	25.4 ± 2.1	0.06
≥7 mm	10.5 ± 2.0	5.7 ± 1.2	9.6 ± 1.5	0.10
CIDR removal				
≥3 mm	18.6 ± 2.5	22.5 ± 2.1	25.3 ± 2.5	0.14
≥7 mm	10.5 ± 2.0[a]	9.1 ± 1.8[a]	18.1 ± 2.7[b]	0.02
12 hours after second AI				
≥3 mm	15.8 ± 4.2	15.8 ± 3.5	13.33 ± 2.3	0.88
≥7 mm	5.4 ± 1.6	9.0 ± 2.1	5.2 ± 1.5	0.20
Estimated number (%) of ovulations between CIDR removal and 12 hours after second AI	5.1 (71.3%)[ab]	0.1 (1.1%)[a]	12.9 (48.6%)[b]	0.02

[a,b]- Within a row, groups without a common superscript differed (P<0.05)

Plasma hormone concentrations

For plasma progesterone concentrations there was only a significant effect of time (Figure 2); concentrations were very low at the time of pLH treatment. However, for plasma LH concentrations, there was an effect of time and a group by time interaction. The long P_4 group had a significantly higher plasma LH concentration compared to the short P_4 group at the time of pLH treatment (Figure 3).

DRAFT Confidential

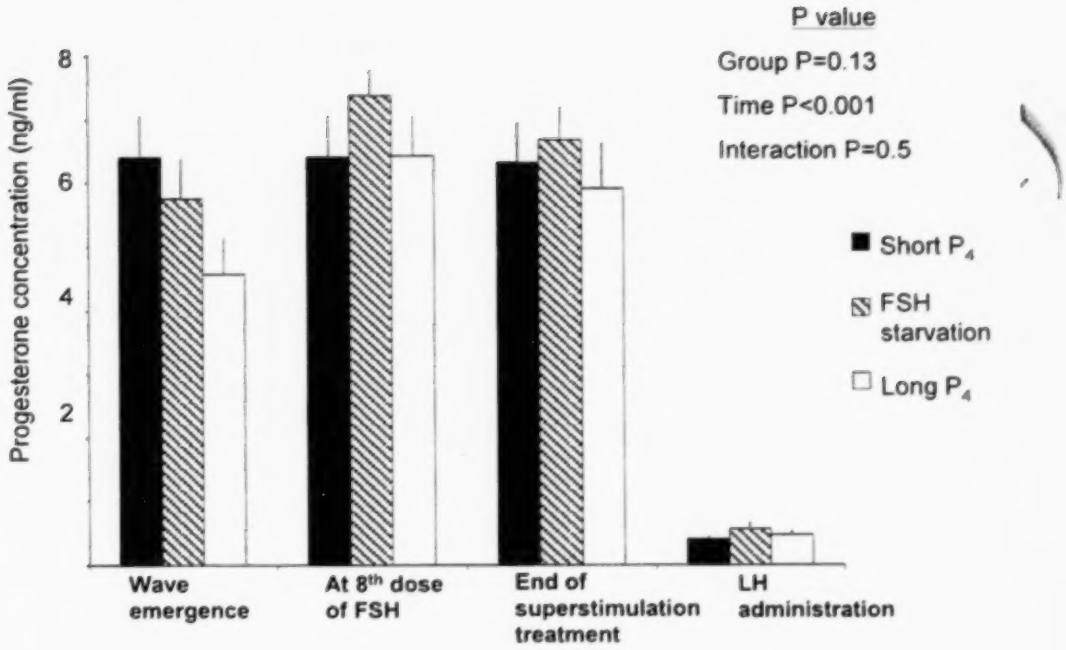


Figure 2 Mean (± SEM) plasma progesterone concentrations in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment. [a,b]- Values without a common superscript were different (P<0.05)

DRAFT

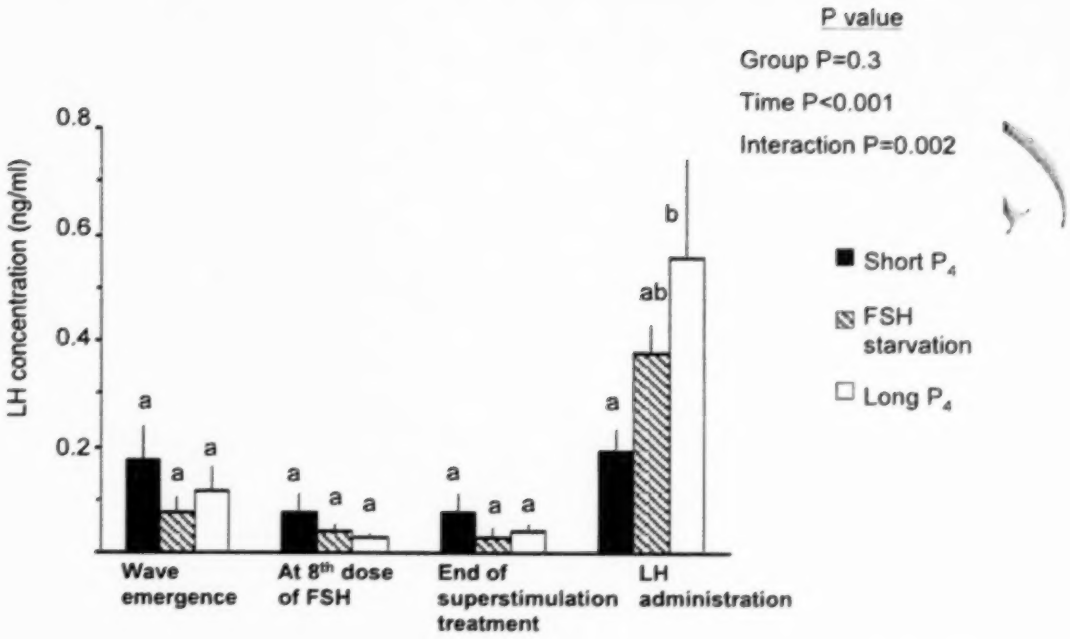


Figure 3 Mean (± SEM) plasma LH in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment. [a,b]- Values without a common superscript were different (P<0.05)

Ovulation and ova/embryo recovery

In the FSH starvation group, 6 of 13, and in the long P_4 group, 1 of 13 cows failed to ovulate (no follicles disappeared between the CIDR removal and 12 hours after the second AI and no new CL detected at slaughter) and were excluded from further analysis. Fewer ovulations were detected in the FSH starvation group than either the short P_4 or long P_4 groups (P=0.001; Table 2), whereas there was no significant difference between the latter two groups.

Most of the embryos were recovered from oviduct. Nine ova/embryos were collect from the uterus of one cow in the short P_4 group, whereas 23 ova/embryos were collected from the uteri of three cows in the long P_4 group. Total recovery rate varied among groups, with the lowest recovery rate in the FSH starvation group (Table 2). In the Short P_4 and FSH starvation groups, the majority of the embryos were Grades 1 or 2, whereas the majority of embryos in the Long P_4 group were Grades 3 or 4 (Table 2).

DRAFT

Table 2 Mean (±SEM) numbers of ovulations and ova/embryos recovered at slaughter 4 days after insemination in cows subjected to a short or long progesterone exposure, or FSH starvation, during superstimulatory treatment.

	Short P_4	FSH starvation	Long P_4	P value
No. cows	14	13	13	-
No. ovulations	11.6 ± 2.2^a	2.5 ± 1.2^b	15.4 ± 3.0^a	0.001
Total ova/embryos recovered	5.9 ± 1.3^a	0.9 ± 0.6^b	6.7 ± 1.5^a	0.048
Recovery rate (%)	50.9^a	36.0^b	43.5^a	0.048
No. uncleaved ova (%)	13/82 (15.9)	1/12 (8.3)	5/87 (5.7)	0.09
No. cleaved ova (%)	69/82 (84.1)	11/12 (91.7)	82/87 (94.3)	0.09
Grades 1 or 2 embryos (%)	87.0^a	81.8^a	37.8^b	0.0001
Grades 3 or 4 embryos (%)	13.0^a	18.2^a	62.2^b	0.0001

[a,b]- Within a row, values without a common superscript differed ($P<0.05$).

There was no significant difference between Short P_4 and Long P_4 groups for embryo development at 7, 9, or 10 days after insemination (Table 3). Results from FSH starvation were excluded, due to a paucity of embryos.

Table 3 Embryo development in culture in cows subjected to a short or long progesterone exposure during superstimulatory treatment.

Interval after AI	Short P_4	Long P_4	P value
7 days			
≥ Morulas	17/69 (24.6%)	15/82 (18.3%)	0.39
Grades 1 or 2 morulas	0/69 (0%)	2/82 (2.4%)	0.19
Grades 3 or 4 morulas	17/69 (24.6%)	13/82 (15.8%)	0.19
9 days			
Early blastocyst	10/69 (14.5%)	16/82 (19.5%)	0.41
Blastocyst	9/69 (13.0%)	15/82 (18.3%)	0.38
≥ Expanded blastocyst	4/69 (5.8%)	7/82 (8.5%)	0.52
Transferable embryos	7/69 (10.1%)	14/82 (17.1%)	0.22
10 days			
≥ Early blastocyst	9/69 (13.0%)	15/82 (18.3%)	0.38
≥ Blastocyst	9/69 (13.0%)	16/82 (19.5%)	0.29
≥ Expanded blastocyst	8/69 (11.6%)	9/82 (11.0%)	0.90
Transferable embryos	7/69 (10.1%)	14/82 (17.1%)	0.22

DRAFT Confidential

Discussion

Oocytes in follicles that grew and developed under a prolonged period of progesterone exposure during superstimulation did not have compromised competence. In that regard, both Short P_4 and Long P_4 groups (analogous to 3-wave and 2-wave cycles, respectively) were similarly capable of producing equal numbers of healthy transferable embryos. Therefore, the hypothesis that oocyte competence in superstimulated cows is improved by short-duration progesterone exposure during follicular growth was not supported. However, FSH starvation led to a substantial reduction in the in the capacity of follicles to ovulate. Therefore, the hypothesis that oocyte competence is not affected by FSH starvation at the end of superstimulatory treatment was not adequately tested.

During the superstimulatory treatment, the long P_4 group had more follicles ≥7 mm at CIDR removal than the FSH starvation group; this was attributed to the difference in total dose of FSH (350 versus 200 mg, respectively). The effect of different total dose of FSH (from 100 to 900 mg) in a superstimulatory treatment has been tested and in many reports, increasing the total dose (up to ~400 mg) increased superovulatory response [26, 78, 126]. Furthermore, the greater number of follicles ≥7 mm at CIDR removal in the long P_4 group could also be attributed to a longer growing phase; a longer period of follicular development (e.g. 2-wave cycle) increased follicle diameter [5, 6, 127]. Most follicles ≥7 mm in diameter at CIDR removal were expected to ovulate within 48 hours after LH administration [3]; this accounted for the need for a third AI in most of the cows (i.e. 3 inseminations at 24, 36, and 48 hours, respectively, after LH treatment).

Based on follicle disappearance between CIDR removal and 12 hours after the second AI and the number of CL after slaughter, 72 hours of FSH starvation at the end of superstimulatory treatment decreased ovulatory response. This was in agreement with another study, in which 132 hours of FSH starvation decreased ovulation rates; however the FSH starvation was created under a prolonged low progesterone environment, inducing a large, persistent ovulatory follicle [2, 6]. It was not clear if the low ovulation rates in that study were caused by the development of persistent follicles.

Follicles in the FSH starvation and long P_4 groups were both under prolonged progesterone exposure, which could have resulted in an aged oocyte. In addition, the FSH starvation group had little FSH available to maintain follicle growth, which resulted in a reduced superstimulatory response, as demonstrated by fewer follicles ≥7 mm at CIDR removal when compared with long P_4 [128]. Follicle growth is dependent on gonadotrophin support (initially FSH and then LH); without this support, follicles undergo atresia [128]. Therefore, the long P_4 group did not have decreased ovulation rates, as FSH stimulation was continued. However, the long P_4 group had poor embryo quality, perhaps due to delayed LH (secondary to prolonged progesterone exposure) and suppressed preovulatory maturation (prior to the LH surge).

Progesterone concentrations were kept high during superstimulatory treatment to mimic mid-luteal progesterone concentration. However, $PGF_2\alpha$ treatment and CIDR removal resulted in low plasma progesterone concentrations at LH administration; these were needed to create a pre-ovulatory endogenous LH peak, since P_4 has a suppressive effect on pituitary release of LH [12, 20, 22, 80]. In the present study, plasma LH concentrations at the time of administration of exogenous LH were highest in the cows in

DRAFT Copyrighted

the Long P_4 group, and tended to be higher in the FSH starvation group than in the Short P_4 group. Since LH is released by the pituitary in pulses [22], multiple samples at frequent intervals should be collected to ensure that the frequency and amplitude of LH is accurately characterized [22]. In the present study, LH concentrations were determined in only a single sample after plasma progesterone concentrations had declined. Therefore, the accuracy of determining LH release using a single blood sample is decreased; we may have detected only a portion of the LH surge, the peaks of a pulse, or increased basal concentrations of LH release in response to the feedback from follicles under prolonged development. Consequently, the lower LH concentration in the Short P_4 group could be an artifact due to a single blood sample, or it could represent a delayed LH surge.

Reproductive tracts were collected 4 days after the second AI; as expected, most of the embryos were retrieved from the oviducts, since bovine embryos enter the uterus 3 to 4 days after fertilization [129, 130]. Due to so few ovulations in the FSH starvation group, the recovery rate was lower than the other two groups, and there were insufficient numbers of embryos to adequately measure development competence.

At slaughter, the long P_4 group had proportionally more low quality embryos than the short P_4 or FSH starvation groups. Although there were insufficient numbers of embryos to adequately measure development competence in the FSH starvation group, there was no obvious difference in the developmental competence of embryos recovered from cows in the Short or Long P_4 groups. Although this could reflect a latent effect, or an improvement in the competence of embryos in the Long P_4 group, development to expanded or hatched blastocyts was quite low in both the Short and Long P_4 groups, suggesting that effects may be more related to handling during collection (at slaughter), or culture conditions.

In conclusion, although there were differences in the response to superstimulation when the duration of the ovulatory wave was extended 3 days, there was no difference in oocyte competence. Long exposure of superstimulated follicles to progesterone (analogous to a 2-follicular wave cycle) decreased embryo quality 4 days after AI; however, superstimulated follicles exposured to progesterone for short and long intervals were similarly capable of producing healthy embryos that developed similarly in culture. Although FSH starvation treatment led to a loss of ovulatory capability, the hypothesis that FSH starvation at the end of superstimulatory treatment does not affect oocyte competence remains untested.

DRAFT Confidential

Acknowledgements

The project was supported by research grants from Saskatchewan Agriculture Development Fund, Westgen Inc. and the Natural Sciences and Engineering Research Council of Canada. The authors thank Pfizer Animal Health for CIDR inserts, Bioniche Inc. for Folltropin and Schering-Plough Animal Health for Estrumate. The authors thank personnel from the Goodale farm for providing cattle and technical assistance.

Effect of circulating concentrations of progesterone on the developmental competence of oocytes in superovulated cows

R. S. Jaiswal[1], J.Singh[2], H.S. Nagra[2], T. Grafton[2], M.H. Ratto[3], P.S. Malhi[4] and G.P. Adams[2]*

[1]Faculty of Veterinary Medicine, University of Calgary, Alberta, Canada
[2]Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Canada.
[3]Faculty of Veterinary Sciences, Universidad Austral de Chile, Valdivia-Chile
[4]Agriculture Canada, Lethbridge, Alberta, Canada

Short title: Effect of progesterone on oocyte competence

* Corresponding author:
Dr. Gregg P. Adams
Professor, Department of Veterinary Biomedical Sciences
Western College of Veterinary Medicine
52 Campus Drive
University of Saskatchewan
Saskatoon, SK S7N 5B4, Canada
Telephone: 306-966-7411
Fax: 306-966-7405
Email: gregg.adams@usask.ca

DRAFT Confidential

Abstract

The study was designed to determine the effect of progesterone (and hence, LH pulsatility) during superstimulatory treatment on the competence of the oocyte to develop into an embryo. The growing phase immediately after wave emergence (Day 0) was subjected to a relatively high, low, or prolonged-low progesterone environment (n=10/group) through the use of progesterone-releasing intravaginal device (CIDR) and luteolytic dose of prostaglandin. Heifers were given 200 mg FSH divided bid over 4 days beginning on Day 0. The CIDR were removed from low- and high-progesterone groups on Day 3, and from the prolonged-low-progesterone group on Day 8. Heifers were given LH 24 hours after CIDR removal, inseminated artificially at 24 and 36 hours after LH, and slaughtered 3 days after insemination. Plasma progesterone was elevated in the high-progesterone group ($P<0.01$), and plasma LH pulse frequency was elevated in low- and prolonged-low-progesterone groups ($P<0.01$). Multiple follicles developed in all groups, but failed to ovulate in the prolonged-low-progesterone group. A higher total recovery rate in the high- vs. low-progesterone group (68% vs. 50%, $P<0.01$) was associated with the collection of a greater number of unfertilized oocytes. However, no differences between the high- and low-progesterone groups were detected in the number of ovulations or in the number of embryos that developed to the 2- to 16-cell stages. We conclude that the progesterone environment (LH pulse-frequency) during the early growing phase after wave emergence does not influence oocyte competence in superstimulated heifers. Results provide rationale for the hypothesis that prolonged exposure to high LH, without continued FSH support, permits continued follicular growth but a loss of ovulatory capability.

Introduction

Results of studies in cattle support the hypothesis that the competence of the oocyte to become fertilized and develop into an embryo is associated with the developmental status of the follicle that contains it (Salamone *et al.*1999; Vassena *et al.*2003). Endocrine events during the preovulatory period ultimately result in the resumption of meiosis and final stages of maturation of the oocyte within the extant dominant follicle (Moor *et al.*1981). Final maturation involves disruption of gap junctions between the oocyte and its cumulus cell investment resulting in a loss of cell-to-cell communication, including the transport of meiosis-inhibiting substance (Larsen *et al.*1986; Motlik *et al.*1986; Wert & Larsen 1990). High circulating concentrations of estradiol in cattle have been associated with a disruption of gap junctions between the oocyte and cumulus cells (Mihm *et al.*1994a; Revah & Butler 1996; Austin *et al.*1999), and high circulating concentrations of LH in rats have been shown to disrupt the gap junctions between the cumulus and mural granulosa cells (Wert & Larsen 1990).

The effects of differing circulating concentrations of progesterone on the development of the dominant follicle in cattle have been attributed to changes in LH secretion. Progesterone suppressed the growing phase of the dominant follicle in a dose-dependent manner (Adams *et al.* 1992), and was associated with suppression of LH pulse-frequency (Ireland & Roche 1982; Roberson *et al.* 1989; Stock & Fortune 1993). Subluteal-phase concentrations of progesterone resulted in elevated LH pulse-frequency (van Cleef *et al.* 1992; Kojima *et al.* 2003) and the development of a persistent, over-sized, estrogen-active dominant follicle (Sirois & Fortune 1990; Adams *et al.* 1992; Savio *et al.* 1993b; Stock & Fortune 1993; Revah & Butler 1996; Kojima *et al.* 2003) while still inhibiting the pre-ovulatory LH surge (Hobson & Hansel 1972). By comparison, luteal-phase concentrations of progesterone resulted in a reduction in LH pulse-frequency (Rahe *et al.* 1980), suppression of the dominant follicle, and a shorter interwave interval (Adams *et al.* 1992; Lucy *et al.* 1992).

Persistent, oversized dominant follicles resulting from subluteal-phase progesterone concentrations (e.g., prolonged progestagen treatment for estrus synchronization) have been associated with poor fertility (Hansel *et al.* 1961; Hill *et al.* 1971; Henricks *et al.* 1973; Roche 1974; Beal *et al.* 1988; Jochle 1993; Savio *et al.* 1993b; Stock & Fortune 1993). The uterine environment (Wehrman *et al.* 1997) and circulating concentrations of progesterone (Stock & Fortune 1993) were not affected following ovulation of a persistent follicle; hence, lower fertility may be attributed to altered oocyte maturation under prolonged exposure to a high estrogen environment (Sirois & Fortune 1990; Savio *et al.* 1993a; Stock & Fortune 1993; Wehrman *et al.* 1993; Mihm *et al.* 1994a) consequent to elevated LH pulse-frequency (Roberson *et al.* 1989; Savio *et al.* 1993a). In this regard, infertility associated with prolonged follicular dominance was attributed to extended period of high LH pulse-frequency resulting in an extended period of oocyte maturation before ovulation (Revah & Butler 1996), and the formation of abnormal (Mihm *et al.* 1994b; Revah & Butler 1996) or retarded embryos that fail to reach the 16-cell stage (Ahmad *et al.* 1995).

Variability in the response to superovulatory treatment, and recovery of fewer than 40% of oocytes that are developmentally competent (Betteridge 2006; Lonergan 2007) continues to limit the utility of embryo transfer technology. Although the influence of progesterone on the growth of the dominant follicle (Adams *et al.* 1992) and oocyte/embryo collection rates (Saumande & Batra 1985; Savio *et al.* 1991; Mapletoft *et al.* 2002) have been examined in detail, the

Proof Copy

relationship between progesterone and the competence of the oocyte contained in the dominant follicle has not.

The objective of the present study was to determine the effect of progesterone (and hence, LH pulsatility) during the final stages of oocyte maturation on the competence of the oocyte to develop into an embryo. The experiment was designed to create a high progesterone, low progesterone, and prolonged-low progesterone environment during the growing phase of the dominant (ovulatory) follicle.

Results

Hormonal profile

In accordance with the induced progesterone milieu, plasma concentrations of progesterone on Day 3 and on the day before AI (Day 4 in high- and low-progesterone groups, and Day 9 in the prolonged-low-progesterone group) were higher in the high-progesterone group compared to the other groups ($P<0.01$; Fig. 1). Within groups, circulating progesterone concentrations declined ($P<0.01$) within 24 hours of CIDR removal.

The mean concentration, basal concentration and pulse amplitude of plasma LH on the day before CIDR removal did not differ among groups; however, LH pulse frequency was lowest ($P<0.05$) in the high-progesterone group (Table 1). Mean plasma LH concentration on Day 3 did not differ among groups ($P=0.37$; Fig. 2). Within groups, mean plasma LH concentration on the day before insemination (i.e., Day 4 in high- and low-progesterone groups and Day 9 in the prolonged-low-progesterone group) was higher ($P<0.01$) than on Day 3 (Fig. 2).

Change in the plasma concentrations of estradiol-17β from Day 3 to the day before insemination (i.e., Day 4 in high- and low-progesterone groups and Day 9 in the prolonged-low-progesterone group) is illustrated in Fig. 3. A treatment-by-group interaction ($P<0.01$) on circulating concentrations of estradiol-17β resulted from an increase ($P<0.05$) from Day 3 to the day before AI in all but the prolonged-low-progesterone group.

Follicular profile

Comparison of follicle numbers in different size categories on the day before insemination (i.e., Day 4 in high- and low-progesterone groups and Day 9 in the prolonged-low-progesterone group) is illustrated in Fig. 4. Heifers in the prolonged-low-progesterone group had more follicles ≥9 mm ($P=0.03$) than heifers in the other groups. There was no difference ($P=0.39$) among groups in the total number of follicles or in the number of follicles <9 mm in diameter.

Oocyte/embryo collection

No corpora lutea were detected and no oocytes/embryos were collected from heifers in the prolonged-low-progesterone group; hence, data from this group were excluded from analyses related to oocyte-embryo collection. There was no difference ($P=0.83$) in the mean number of ovulations detected per heifer at the time of slaughter between the high- and low-progesterone groups (Table 2). The proportion of oocytes plus embryos collected per number of ovulations was higher ($P<0.01$) in the high- than in the low-progesterone group as a result of a greater number ($P<0.01$) of unfertilized oocytes in the latter (Table 2). No difference between groups

DRAFT (Confidential)

was detected in the total number of embryos collected (P=0.39) or in the proportion of embryos at the respective stages of development (Table 2); however, the proportion of 16-cell embryos tended to be higher (P=0.09) in the low progesterone group. A greater proportion of oocytes and embryos were recovered from the uterine tube vs. uterine horn in the high- than in the low-progesterone group (P<0.01; Table 2).

Discussion

The present study was designed to compare the developmental competence of oocytes from dominant follicles that developed under a short-term high- vs. short-term low-progesterone environment and under an extended period of development in a low-progesterone environment (i.e., similar to that of a persistent dominant follicle). The treatment protocol was effective in inducing the intended hormonal milieu. Circulating progesterone concentrations were maintained at sub-luteal phase levels in the low progesterone groups and LH pulse-frequency varied inversely to progesterone, in accordance with the previous studies (Ireland & Roche 1982; Roberson *et al.* 1989; Sirois & Fortune 1990; Savio *et al.* 1993a; Stock & Fortune 1993; Custer *et al.* 1994; Kinder *et al.* 1996; Binelli *et al.* 1999; Vinoles *et al.* 1999).

Complete failure to ovulate in the prolonged-low progesterone group was unexpected and prevented evaluation of oocyte competence in this group. Although the ovarian superstimulatory response was similar to the other treatment groups, none of the follicles ovulated despite exogenous LH treatment. Although individual follicle diameter profiles were not recorded, the prolonged growing and static phase of the dominant follicle induced by the extended period of low progesterone treatment was apparent by a significantly greater number of follicles ≥ 9 mm at the time of LH treatment (5 days after the end of FSH treatment) compared to short-term progesterone groups (LH given 1 day after the end of FSH treatment). Oversized follicles (≥ 20 mm) were not detected, but were not expected in a superstimulated model where multiple follicles compete for available LH (Adams *et al.* 1993), secrete lower concentrations of estradiol (Revah & Butler 1996), and remain smaller in size than single dominant follicles (Adams *et al.* 1993; Revah & Butler 1996). A period of 5 days without FSH support, despite elevated LH pulse-frequency, resulted in follicular atresia, evinced by low circulating concentrations of estradiol (Lucy *et al.* 1992; Savio *et al.* 1993a) and the loss of ovulatory capability. Circulating concentrations of progesterone were not elevated at the time of LH treatment; hence, follicular atresia was not associated with luteinization in the prolonged-low progesterone group.

The recovery rate of oocytes/embryos (number of oocytes plus embryos collected per total number of ovulations detected) was higher in the high- vs. the low-progesterone group. The reason for this is unclear, but may be associated with greater efficiency of collection from the uterine tube than from the uterine horn. Under a low-progesterone milieu, oocytes/embryos were transported to the uterine horns faster than those that developed under a high-progesterone milieu, as reflected in the higher proportion collected from the uterine tubes rather than the uterine horn in the high-progesterone group. Estradiol facilitates the transport of oocytes and embryos towards the uterus by peristaltic contractions of the uterine tubes (Ingersoll 1962). In contrast, progesterone suppresses the frequency and amplitude of uterine tube contractions (Maia & Coutinho 1970). The lower oocyte-embryo collection rate in the low-progesterone group may be attributed to a more rapid rate of transport from the uterine tubes to the uterine horns under the influence of high estradiol, and lower collection efficiency from the uterine horn.

DRAFT (Unedited)

The day of embryo collection in the present study was selected based on the finding that in superovulated cows, the majority of oocytes/embryos were collected from the uterine tubes 3 days after the 2nd insemination (Holy *et al.* 1992). Hence, the relatively high collection rate from the uterine horns on Day 2 (or 3 days after insemination) in the present study was unexpected.

Although collection efficiency was higher in the high-progesterone group, the difference was attributed to the collection of a greater number of unfertilized oocytes. The proportion per ovulation of embryos at various stages of development, and collectively, was similar between groups. Alternatively, a lower proportion of unfertilized oocytes in the low-progesterone group may be interpreted as an improved fertilization capacity. High LH pulse-frequency (i.e., low progesterone) stimulates estradiol production (Desaulniers *et al.* 1995) by making aromatizable substrate available for the production of estradiol by the granulosa cells of developing follicles (Lucy *et al.* 1992). If such an effect on fertilization capacity exists, it may arise from a direct effect of LH and estradiol on oocyte maturation within the follicular antrum or from the effect on the tubal environment. High estradiol is associated with the secretion of oviductal proteins and oviductal epithelial cell proliferation (Slayden & Brenner 1994), and makes the uterine environment conducive to efficient gamete transport (Fuentealba *et al.* 1988), sperm capacitation (Anderson & Killian 1994), sperm-egg binding (Staros & Killian 1998), and fertilization (Binelli *et al.* 1999). Receptors for LH are expressed in the uterine tube and tubal proteins are secreted in a dose-dependent manner in response to treatment with hCG (Sun *et al.* 1997); hence, the effect of high-estradiol-low-progesterone environment may be mediated through the resulting high concentrations of LH.

In summary, short-term (3 days) exposure to high- (luteal) vs. low- (sub-luteal) circulating concentrations of progesterone during the growing phase of the ovulatory follicle in superstimulated heifers influenced LH pulse frequency, but was not associated with an alteration in the competence of oocytes to develop into embryos *in vivo*. Oocyte-embryo collection relative to the number of ovulations was greater in superstimulated heifers in the high- (luteal) vs. low- (sub-luteal) progesterone groups, and was attributed to greater efficiency of recovery from the oviducts compared to the uterine horn in the respective groups. Prolonged exposure (8 days) to sub-luteal phase concentrations of progesterone (and high LH pulse-frequency) resulted in the loss of ovulatory capability in superstimulated heifers.

Materials and Methods

Animals & Treatments

The experiment was done between June and August, and the cattle were maintained in outdoor corrals at the University of Saskatchewan Goodale Research Farm (52°N and 106°W). Hereford crossbred heifers (n = 49), 20 to 24 months of age and weighing from 480 to 680 kg, were treated with a single luteolytic dose of cloprostenol (500 µg Estrumate, Schering-Plough Animal Health, Pointe-Claire, Quebec, Canada) intramuscularly. The ovaries were examined daily by transrectal ultrasonography using a B-mode ultrasound scanner with a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan). The first 30 heifers that ovulated were assigned randomly to one of three groups (n=10 heifers per group) designed to induce high-, low-, or prolonged-low-circulating concentrations of progesterone (Fig. 5).

To minimize and synchronize the interval to new wave emergence among heifers, transvaginal ultrasound-guided ablation of all follicles ≥5 mm was done between 5 and 8 days

DRAFT Copyright

after ovulation (Bergfelt *et al.* 1994). Heifers in the low- and prolonged-low-progesterone groups were treated with two luteolytic doses of cloprostenol 12 hours apart on the day of follicular ablation, whereas heifers in the high-progesterone group were not given prostaglandin so that their CL remained functional. A once-used (i.e., partially spent) intravaginal progesterone-releasing device (1.9 g progesterone; CIDR-B, Bioniche Animal Health, Belleville, Ontario, Canada) was inserted immediately after follicular ablation to maintain sub-luteal phase plasma concentrations of progesterone in the low-progesterone groups (van Cleef *et al.* 1992) and to supplement endogenous progesterone in the high-progesterone group. Beginning 24 hours post-ablation (i.e., expected time of new follicular wave emergence, Day 0; Bergfelt *et al.* 1994), heifers were given 25 mg of pFSH intramuscularly every 12 hours for 4 days (total dose of 200 mg Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada), which is half the standard superstimulatory dose for cattle (Looney *et al.* 1988; Wilson *et al.* 1989). On Day 3, heifers in the high-progesterone group were given a luteolytic dose of cloprostenol in the morning and in the evening, and the CIDR device was removed from heifers in the low- and high-progesterone groups in the evening. The CIDR device was removed from heifers in the prolonged-low-progesterone group on the evening of Day 8. All heifers were given a single intramuscular dose of pLH (25 mg Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada) 24hours after CIDR removal to induce ovulation. Heifers were artificially inseminated 24 and 36 hours after LH treatment by one inseminator using frozen semen of the same ejaculate of a single bull. The heifers were sent to slaughter at 108 hours post-LH treatment to recover oocytes/embryos from the excised reproductive tracts. Follicular development and ovulation were monitored by transrectal ultrasonography on Day -1 (day of follicle ablation), Day 4, and on the day before heifers were sent to slaughter. In addition, the ovaries of heifers in the prolonged-low-progesterone group were examined by ultrasonography on Day 9.

Embryo collection

Reproductive tracts were transported at ambient temperature to the laboratory in plastic bags within 3 hours of slaughter. The number of corpora lutea in each ovary was recorded as an estimate of the number of ovulations. The uterine tubes were sectioned about 1 cm distal to the utero-tubal junction, to include a small part of the tip of the uterine horn, and were dissected from the mesosalphinx to straighten them. Each uterine tube was flushed with 20 ml of phosphate buffered saline (supplemented with 0.4% BSA) by inserting a blunt 18 gauge needle into the infundibulum; flushed material was collected in petri dishes (100 x 15 mm; Becton Dickinson, Franklin Lakes, NJ) at the utero-tubal junction. The remaining part of the uterine horn was dissected from the mesometrium and the base of each horn (near the bifurcation) was ligated with large tissue forceps. A stab-incision was made near the base of the ligated uterine horn to permit insertion of the tip of a 60 ml catheter-tipped syringe and infusion of 75 ml of flushing medium. The flush was collected in petri dishes at tip of the uterine horn. Medium flushed from the uterine tubes and horns was passed through a 70 micron filter apparatus (Emcon®, Veterinary Concepts, Spring Valley, WI) and oocytes/embryos were evaluated using a stereo-microscope.

Blood sampling and radioimmuno-assays

DRAFT Confidential

To estimate circulating LH, estradiol, and progesterone concentrations, jugular blood samples were collected in heparinized tubes (10 ml; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) on Day -1, Day 3, and immediately before LH treatment (i.e., Day 4 in high- and low-progesterone groups, and Day 9 in the prolonged-low-progesterone group). To compare LH pulse characteristics under different progestational environments, blood samples were collected at 15-min intervals for 8 hours (n = 4 heifers per group) starting at 36 hours before CIDR removal (i.e., on Day 2 in the high- and low-progesterone groups, and on Day 7 in the prolonged-low-progesterone group). Samples (5 ml) were collected via an intravenous catheter (vinyl tubing; 1.0 mm inner diameter × 1.5 mm outer diameter; Product code SV70; Critchley Electrical Products Pty Ltd., Auburn, NSW, Australia) that was placed in the jugular vein on the day before serial sampling to minimize the effect of handling stress on plasma gonadotropin concentrations. The catheter was filled with heparinized saline between sampling (1000 U.S.P. units of sodium heparin per liter of saline; Hepalean, Organon Teknika Inc., Toronto, Ontario, Canada). Blood samples were centrifuged for 15 minutes at 1500 x g within 60 minutes of collection, and the plasma was stored at -20°C.

Plasma concentrations of LH were measured using a double antibody radioimmunoassay, and were expressed as NIDDK-bLH4 units (Evans *et al.* 1994; Honaramooz *et al.* 1998). The range of the standard curve was 0.06 to 8 ng/ml. The intra-assay coefficients of variation were 7% for low reference sera (mean, 0.43 ng/ml) and 2% for high reference sera (mean, 0.99 ng/ml). The LH pulse frequency, pulse amplitude, mean, and basal concentrations were calculated as described previously (Honaramooz *et al.* 1998).

Plasma concentrations of progesterone were determined (Kastelic *et al.* 1999) using a solid-phase radioimmunoassay (catalogue # TKPG5; Lot # TKPG21443; Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA). The range of the standard curve was 0.1 to 40 ng/ml. The intra-assay coefficients of variation were 6% for low reference sera (mean, 1.5 ng/ml), 8% for medium reference sera (mean, 2.6 ng/ml), and 3% for high reference sera (mean, 15.0 ng/ml).

Plasma concentrations of estradiol-17β were measured using a double antibody radioimmunoassay (Joseph *et al.* 1992) (Catalog # KE2D5; Diagnostics Products Corporation, Los Angeles, CA). The range of the standard curve was 2 to 500 pg/ml. The intra-assay coefficients of variation were 2% and 4% for samples with means of 28.3 and 94.9 pg/ml, respectively.

Statistical analyses

Statistical analyses were performed using the Statistical Analysis System software package (SAS learning edition 2.0; SAS Institute Inc., Cary, NC, USA). Proportions of oocytes and embryos collected and the proportions of embryos that developed to successive stages were compared between groups by chi-square analyses. The effect of treatment (high-, low-, and prolonged-low-progesterone) on the plasma LH profile (mean and basal concentrations, pulse frequency and amplitude) was compared by analysis of variance using the General Linear Model procedure (proc GLM). The effect of treatment and day on hormone concentrations and the number of follicles in different diameter categories were determined by analysis of variance for repeated measures using the mixed procedure (proc Mixed; Littell *et al.* 2000). If main effects or their interaction were statistically significant ($P<0.05$), multiple comparisons were made using Tukey's post-hoc test. The values are expressed in mean ± SEM unless otherwise stated.

DRAFT Confidential

The experimental protocol was approved by the University Committee on Animal Care and Supply, under the guidelines of the Canadian Council on Animal Care.

Acknowledgements

The project was supported by research grants from Saskatchewan Agriculture Development Fund, Westgen Inc. and the Natural Sciences and Engineering Research Council of Canada. We thank Bioniche Animal Health Canada Inc. for providing Folltropin-V, Lutropin and CIDR devices, and Schering-Plough Animal Health, Canada for providing Estrumate.

References

Adams GP, Kot K, Smith CA & Ginther OJ 1993 Selection of a dominant follicle and suppression of follicular growth in heifers. *Animal Reproduction Science* **30** 259-271.

Adams GP, Matteri RL & Ginther OJ 1992 Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. *Journal of Reproduction and Fertility* **95** 627-640.

Ahmad N, Schrick FN, Butcher RL & Inskeep EK 1995 Effects of persistent follicles on early embryonic losses in beef cows. *Biology of Reproduction* **53** 1129-1135.

Anderson SH & Killian GJ 1994 Effect of macromolecules from oviductal conditioned medium on bovine sperm motion and capacitation. *Biology of Reproduction* **51** 795-799.

Austin EJ, Mihm M, Ryan MP, Williams DH & Roche JF 1999 Effect of duration of dominance of the ovulatory follicle on onset of estrous and fertility in heifers. *Journal of Animal Science* **77** 2219-2226.

Beal WE, Chenault JR, Day ML & Corah LR 1988 Variation in conception rates following synchronization of estrus with melengesterol acetate and prostaglandin F2α. *Journal of Animal Science* **66** 599-602.

Bergfelt DR, Lightfoot KC & Adams GP 1994 Ovarian synchronization following ultrasound-guided transvaginal follicle ablation in heifers. *Theriogenology* **42** 895-907.

Betteridge KJ 2006 Farm Animal Embryo Technologies: Achievements and Perspectives. *Theriogenology* **65** 905-913.

Binelli M, Hampton J, Buhi WC & Thatcher WW 1999 Persistent dominant follicle alters pattern of oviductal secretory proteins from cows at estrus. *Biology of Reproduction* **61** 127-134.

Custer EE, Beal WE, Wilson SJ, Meadows AW, Berardinelli JG & Adair R 1994 Effect of melengesterol acetate (MGA) or progesterone-releasing intravaginal device (PRID) on follicular development, concentrations of estradiol-17 beta and progesterone, and luteinizing

DRAFT Confidential

hormone release during an artificially lengthened bovine estrous cycle. *Journal of Animal Science* **72** 1282-1289.

Desaulniers DM, Lussier JG, Goff AK, Bousquet D & Guilbault LA 1995 Follicular development and reproductive endocrinology during and after superovulation in heifers and mature cows displaying contrasting superovulatory responses. *Domestic Animal Endocrinology* **12** 117-131.

Evans ACO, Adams GP & Rawlings NC 1994 Endocrine and ovarian follicular changes leading up to the first ovulation in prepubertal heifers. *Journal of Reproduction and Fertility* **100** 187-194.

Fuentealba B, Nieto M & Croxatto HB 1988 Estrogen and progesterone receptors in the oviduct during egg transport in cyclic and pregnant rats. *Biology of Reproduction* **39** 751-757.

Hansel W, Malven PV & Black DL 1961 Estrous cycle regulation in the bovine. *Journal of Animal Science* **20** 621-625.

Henricks DM, Hill JR & Dickey JF 1973 Plasma ovarian hormone levels and fertility in beef heifers treated with melengesterol acetate (MGA). *Journal of Animal Science* **37** 1169-1175.

Hill JR, Lamond DR, Henricks DM, Dickey JF & Niswender GD 1971 The effect of melengesterol acetate (MGA) on ovarian function and fertilization in beef heifers. *Biology of Reproduction* **4** 16-22.

Hobson WC & Hansel W 1972 Plasma LH levels after ovariectomy, corpus luteum removal and estradiol administration in cattle. *Endocrinology* **91** 185-190.

Holy L, Vanatka F & Lopatarova M 1992 Descent of ova and embryos in cows superovulated with serum gonadotropin. *Veterinary Medicine (Praha)* **37** 353-364.

Honaramooz A, Chandolia RK, Beard AP & Rawlings NC 1998 Excitatory amino acid regulation of gonadotropin secretion in prepubertal heifer calves. *Biology of Reproduction* **59** 1124-1130.

Ingersoll FM 1962 The fallopian tubes. *Clinical Obstetrics and Gynecology* **5** 779.

Ireland JJ & Roche JF 1982 Effect of progesterone on basal LH and episodic LH and FSH secretion in heifers. *Journal of Reproduction and Fertility* **64** 295-302.

Jochle W 1993 Forty years of control of the oestrous cycle in ruminants progress made, unresolved problems and the potential impact on sperm encapsulation technology. *Reproduction Fertility and Development* **5** 587-594.

DRAFT Confidential

Joseph IB Currie WD & Rawlings NC 1992 Effects of time after ovariectomy, season and oestradiol on luteinizing hormone and follicle-stimulating hormone secretion in ovariectomized ewes. *Journal of Reproduction and Fertility* **94** 511-523.

Kastelic JP, Olson WO, Martinez M, Cook RB & Mapletoft RJ 1999 Synchronization of estrus in beef cattle with norgestomet and estradiol valerate. *Canadian Veterinary Journal* **40** 173-178.

Kinder JE, Kojima FN, Bergfelt EG, Wehrman ME & Fike KE 1996 Progestins and estrogen regulation of pulsatile LH release and development of persistent ovarian follicles in cattle. *Journal of Animal Science* **74** 1424-1440.

Kojima FN, Bergfeld EG, Wehrman ME, Cupp AS, Fike KE, Mariscal-Arroyo DV, Sanchez-Torres T, Garcia-Winder M, Clopton DT, Roberts AJ & Kinder JE 2003 Frequency of luteinizing hormone pulses in cattle influences duration of persistence of dominant ovarian follicles, follicular fluid concentrations of steroids, and activity of insulin-like growth factor binding proteins. *Animal Reproduction Science* **77** 187-211.

Larsen WJ, Wert SE & Brunner GD 1986 A dramatic loss of cumulus cell gap junctions is correlated with germinal vesicle breakdown in rat oocytes. *Developmental Biology* **113** 517–521.

Littell, RC Pendergast J & Natarajan R 2000 Modelling covariance structure in the analysis of repeated measures data. *Statistics in Medicine* **19** 1793-1819.

Lonergan P 2007 State-of-the-art embryo technologies in cattle. *Society for Reproduction & Fertility* Supplement **64** 315-325.

Looney CR, Bondioli KR, Hill KG & Massey JM 1988 Superovulation of donor cows with bovine follicle-stimulating hormone (bFSH) produced by recombinant DNA technology. *Theriogenology* **29** 271 (abstract).

Lucy MC, Savio JD, Badinga L, de-la-Sota RL & Thatcher WW 1992 Factors that affect ovarian follicular dynamics in cattle. *Journal of Animal Science* **70** 3615-3626.

Maia HS & Continho EM 1970 Peristalsis and antiperistalsis of the human fallopian tube during the menstrual cycle. *Biology of Reproduction* **2** 305-314.

Mapletoft RJ, Steward KB & Adams GP 2002 Recent advances in the superovulation in cattle. *Reproduction Nutrition and Development* **42** 601-611.

Mihm M, Baguisi A, Boland MP & Roche JF 1994a Association between the duration of dominance of the ovulatory follicle and pregnancy rate in beef heifers. *Journal of Reproduction and Fertility* **102** 123-130.

DRAFT Confidential

Mihm M, Currant N, Hytell P, Boland MP & Roche JF 1994b Resumption of meiosis in cattle oocytes from pre-ovulatory follicles with a short and long duration of dominance. *Journal of Reproduction and Fertility* Abstract series 13-14.

Moor RM, Osborn JC, Cran DC & Walters DE 1981 Selective effect of gonadotrophins on cell coupling, nuclear maturation and protein synthesis in mammalian oocytes. *Journal of Embryology and Experimental Morphology* **61** 347-365.

Motlik J, Fulka J & Flechon JE 1986 Changes in intercellular coupling between pig oocytes and cumulus cells during maturation in vivo and in vitro. *Journal of Reproduction and Fertility* **76** 31–37.

Rahe CH, Owens RE, Fleeger JL, Newton HJ & Harms PG 1980 Pattern of plasma luteinizing hormone in the cyclic cow dependence upon the period of the cycle. *Endocrinology* **107** 498-503.

Revah I & Butler WR 1996 Prolonged dominance of follicles and reduced viability of bovine oocytes. *Journal of Reproduction and Fertility* **106** 39–47.

Roberson MS, Wolfe MW, Stumpf TT Kittock RJ & Kinder JE 1989 Luteinizing hormone secretion and corpus luteum function in cows receiving two levels of progesterone. *Biology of Reproduction* **41** 997-1003.

Roche JF 1974 Synchronization of oestrus in heifers with implants of progesterone. *Journal of Reproduction and Fertility* **41** 337-344.

Salamone DF, Adams GP & Mapletoft RJ 1999 Changes in the cumulus-oocyte complex of subordinate follicles relative to follicular wave status in cattle. *Theriogenology* **52** 549-561.

Saumande J & Batra SK 1985 Superovulation in the cow: comparison of oestradiol-17 beta and progesterone patterns in plasma and milk of cows induced to superovulate; relationships with ovarian responses. *Journal of Endocrinology* **107** 259-264.

Savio JD, Bonyeen H, Drost M, Lucy MC & Thatcher WW 1991 Follicular dynamics and superovulatory response in Holstein cows treated with FSH-P in different endocrine states. *Theriogenology* **35** 915-929.

Savio JD, Thatcher WW, Badinga L, de la Sota RL & Wolfenson D 1993a Regulation of dominant follicle turnover during the oestrous cycle in cows. *Journal of Reproduction and Fertility* **97** 197-203.

Savio JD, Thatcher WW, Morris GR, Entwistle K, Drost M & Mattiacci MR 1993b Effects of induction of low plasma progesterone concentrations with a progesterone-releasing intravaginal device on follicular turnover and fertility. *Journal of Reproduction and Fertility* **98** 77-84.

DRAFT (Confidential)

Sirois J & Fortune JE 1990 Lengthening the bovine estrous cycle with low levels of exogenous progesterone A model for studying ovarian follicular dominance. *Endocrinology* **127** 916-925.

Slayden OD & Brenner RM 1994 RU 486 action after estrogen priming in the endometrium and oviducts of rhesus monkeys (*Macaca mulatta*). *Journal of Clinical Endocrinology and Metabolism* **78** 440-448.

Staros AL & Killian GJ 1998 In vitro association of six oviductal fluid proteins with the bovine zona pellucida. *Journal of Reproduction and Fertility* **112** 131-137.

Stock AE & Fortune JE 1993 Ovarian follicular dominance in cattle relationship between prolonged growth of the ovulatory follicle and endocrine parameters. *Endocrinology* **132** 1108-1114.

Sun T, Lei ZM & Rao CV 1997 A novel regulation of the (oviductal) glycol-protein gene expression by luteinizing hormone in bovine tubal epithelial cells. *Molecular Cell Endocrinology* **131** 97-108.

van Cleef J, Lucy MC, Wilcox CJ & Thatcher WW 1992 Plasma and milk progesterone and plasma LH in ovariectomized lactating cows treated with new or used controlled internal drug release devices. *Animal Reproduction Science* **27** 91-106.

Vassena R, Mapletoft RJ, Allodi S, Singh J & Adams GP 2003 Morphology and developmental competence of bovine oocytes relative to follicular status. *Theriogenology* **60** 923-932.

Vinoles C, Meikle A, Forsberg M & Rubianes E 1999 The effect of subluteal levels of exogenous progesterone on follicular dynamics and endocrine pattern during the early luteal phase of the ewe. *Theriogenology* **51** 1351-1361.

Wehrman ME Pike KE, Melvin EJ, Kojima FN & Kinder JE 1997 Development of a persistent ovarian follicle and associated elevated concentrations of 17β-estradiol preceding ovulation does not alter the pregnancy rate after embryo transfer in cattle. *Theriogenology* **47** 1413-1421.

Wehrman ME, Roberson MS, Cupp AS, Kojima FN, Stumpf TT, Werth LA, Wolfe MW, Kittok RJ & Kinder JE 1993 Increasing exogenous progesterone during synchronization of estrus decreases endogenous 17β-estradiol and increases conception in cows. *Biology of Reproduction* **49** 214-220.

Wert SE & Larsen WJ 1990 Preendocytotic alterations in cumulus cell gap junction precede meiotic resumption in the rat cumulus-oocyte complex. *Tissue and Cell* **22** 827-852.

DRAFT (Confidential)

Wilson JM, Moore K, Jones AL & Looney CR 1989 Recombinant bovine follicle stimulating hormone: Dose and duration regimens for superovulation of embryo donors. *Theriogenology* **31** 273 (abstract).

DRAFT (Confidential)

548 **Figure captions**
549
550 **Figure 1** Circulating plasma concentrations of progesterone (mean ± SEM) on Day 3 (Day 0 =
551 1st FSH treatment) and on the day before artificial insemination in heifers treated to induce low-,
552 prolonged-low-, and high-progesterone environments. [abcd] Values with no common superscripts
553 were different ($P < 0.05$).
554
555 **Figure 2** Circulating plasma concentrations of LH (mean ± SEM) on Day 3 (Day 0 = 1st FSH
556 treatment) and on the day before artificial insemination in heifers treated to induce low-,
557 prolonged-low-, and high-progesterone environments.
558
559 **Figure 3** Circulating plasma concentrations of estradiol-17β (mean ± SEM) on Day 3 (Day 0 =
560 1st FSH treatment) and on the day before artificial insemination in heifers treated to induce low-,
561 prolonged-low-, and high-progesterone environments. [abc] Values with no common superscripts
562 were different ($P < 0.05$).
563
564 **Figure 4** Number of follicles in different size categories 12 hours before treatment with an
565 ovulatory dose of LH in heifers superstimulated during a low-, prolonged-low-, or high-
566 progesterone environment. [a,b] Within follicle categories, values with no common superscripts are
567 different ($P < 0.05$).
568
569 **Figure 5** Experimental protocol to create a high- (A), low- (B), and prolonged-low- (C)
570 progesterone environment *in vivo*. Follicles ≥5 mm were ablated by transvaginal ultrasound-
571 guided aspiration 5 to 8 days after ovulation to induce new wave emergence. In the presence of a
572 functional CL, a low progesterone device (once-used CIDR) was inserted in the vagina to create
573 a high-progesterone environment (A), whereas, in the absence of CL (PGF2α induced luteolysis),
574 a once-used CIDR was inserted in the vagina to create a low- (B) or prolonged-low- (C)
575 progesterone environment. Heifers in all groups were treated with FSH to obtain more
576 oocytes/embryos for statistical comparison. D: day; D0 = expected day of wave emergence and
577 the first day of FSH treatment; AI: artificial insemination; CIDR: controlled internal drug release
578 device; FSH: follicle stimulating hormone; LH: luteinizing hormone; PGF: prostaglandin F2α;
579 U/S: ultrasonography.
580

DRAFT Confidential

580 **Table 1** Plasma LH pulsatility 36 hours before CIDR removal in heifers treated to induce high-,
581 low-, and prolonged-low-progesterone environments (mean ±SEM).

	High	**Low**	**Prolonged-low**
Mean (ng/ml)	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
Basal (ng/ml)	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}	0.1 ± 0.0^{a}
Pulse amplitude (ng/ml)	0.3 ± 0.1^{a}	0.2 ± 0.1^{a}	0.2 ± 0.0^{a}
Pulse frequency/8 hour	1.8 ± 0.5^{a}	6.0 ± 0.0^{b}	5.3 ± 0.9^{b}

582 [ab] Within rows, values with no common superscript are different ($P < 0.05$).
583

DRAFT (Confidential)

583 **Table 2** Comparison of response to the superstimulatory treatment and rate of ova/embryo
584 transport in the genital tract of heifers under low (n = 10) vs. high-progesterone (n = 10)
585 environments.

End-points		Low progesterone	High progesterone
Ovulations per heifer (CL detected at slaughter; mean ± SEM)		22.3 ± 4.1[a]	20.4 ± 4.7[a]
Total oocytes and embryos collected per ovulation		111/223 (50%)[a]	139/204 (68%)[b]
Unfertilized oocytes collected per ovulation		7/223 (3%)[a]	37/204 (18%)[b]
Embryos collected per ovulation		104/223 (47%)[a]	102/204 (50%)[a]
2-cell embryos		0/104 (0%)[a]	2/102 (2%)[a]
	(mean ± SEM)	0[a]	0.2 ± 0.1[a]
4-cell embryos		8/104 (8%)[a]	13/102 (13%)[a]
	(mean ± SEM)	0.8 ± 0.5[a]	1.3 ±0.6[a]
8-cell embryos		55/104 (53%)[a]	58/102 (57%)[a]
	(mean ± SEM)	5.5 ± 1.1[a]	5.8 ± 2.3[a]
16-cell embryos		41/104 (39%)[a]	29/102 (28%)[a]
	(mean ± SEM)	4.1 ± 1.4[a]	2.9 ± 1.9[a]
Oocytes + embryos recovered from uterine tubes		17/111 (15%)[a]	51/139 (37%)[b]

586 [ab]Values within rows with no common superscript are different ($P < 0.05$)
587

DRAFT (Confidential)

Fig.1: Jaiswal et al.

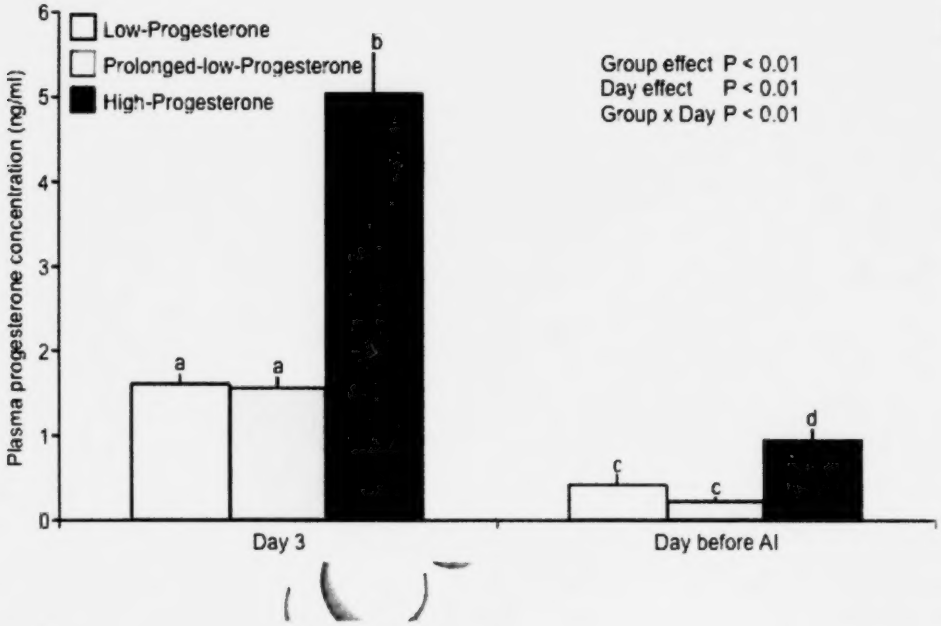


Fig.2: Jaiswal et al.

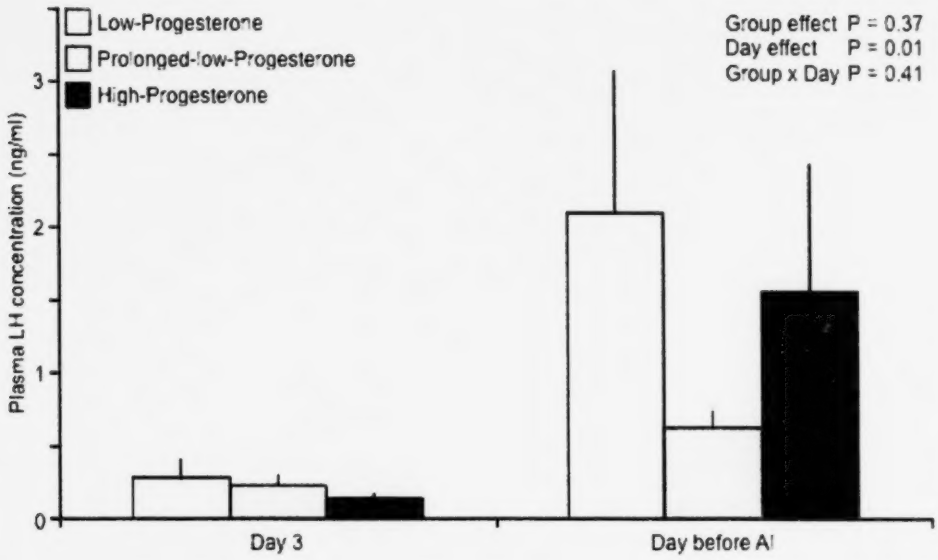
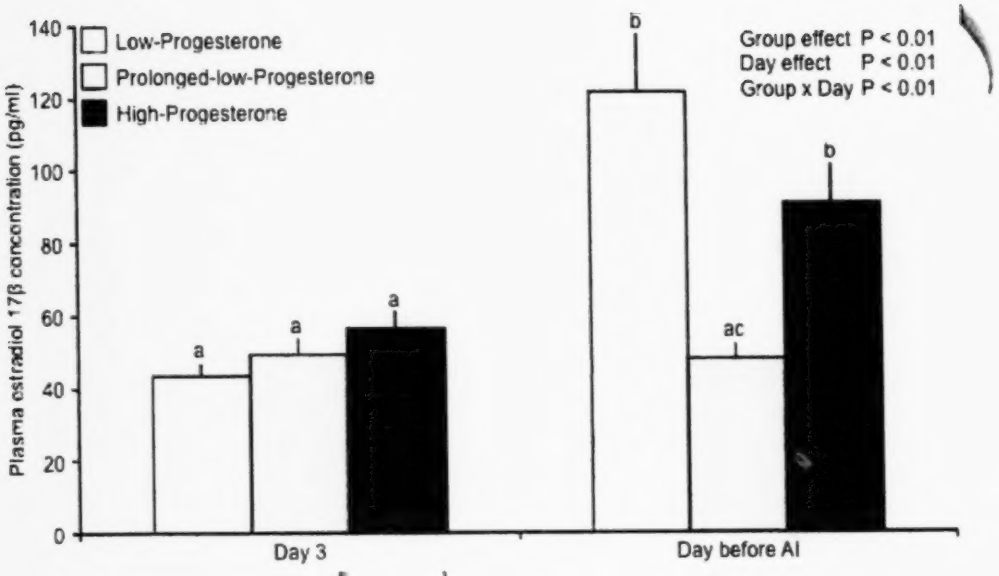


Fig.3: Jaiswal et al.



DRAFT (C

Submitted to Domestic Animal Endocrinology

Progesterone concentration, estradiol pre-treatment, and dose of GnRH affect GnRH-mediated LH release in beef heifers

Dias FCF [1], Colazo MG [2], Kastelic JP [3], Mapletoft RJ [4], Adams GP [1], Singh J [1]*

[1]Department of Veterinary Biomedical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, Canada, S7N 5B4
[2]Dairy Research and Technology Centre, Alberta Agriculture and Rural Development, Edmonton, AB, Canada, T6H 5T6
[3]Agriculture and Agri-Food Canada, Lethbridge Research Centre, Box 3000, Lethbridge, AB, Canada, T1J 4B1
[4]Department of Large Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan, 52 Campus Drive, Saskatoon, Saskatchewan, Canada, S7N 5B4
*Corresponding author

Address for Correspondence:

Dr. Jaswant Singh, Department of Veterinary Biomedical Sciences, University of Saskatchewan, 52 Campus Drive, Saskatoon, SK, S7N 5B4, Canada
Phone: 1-306-966-7410
Fax: 1-306-966-7405
Email: jaswant.singh@usask.ca

DRAFT (Confidential)

Abstract

We hypothesized that increased doses of GnRH or exogenous estradiol (E_2) will enhance GnRH-induced LH release in heifers with elevated plasma progesterone (P_4) concentrations. Two experiments (both 2 x 2 factorial designs) were conducted using nulliparous, postpubertal Angus-cross heifers. In Experiment 1, all heifers (n=32) had their two largest ovarian follicles ablated 5 d after ovulation, and they were given a once-used, intravaginal P_4-releasing insert (CIDR). Furthermore, these heifers were randomly assigned to be given either prostaglandin $F_{2\alpha}$ (immediately and 12 h later; Low-P_4) or no treatment (High-P_4). Six days after emergence of a new ovarian follicular wave (detected by ultrasonography), half of the heifers in each group (n=8) were given either 100 or 200 µg of GnRH. There were significant effects of P_4, GnRH dose, time, and their interactions, on plasma LH concentrations after GnRH treatment; plasma LH concentrations were higher in the Low-P_4 than in the High-P_4 group, and in heifers given 200 vs 100 µg of GnRH (15.4 vs 9.1 and 14.7 vs 9.8 ng/mL, respectively). Ovulation rate was higher (P=0.002) in the Low-P4 group (15/16) than in the High-P4 group (6/16), but GnRH dose had no effect (P=0.4). In Experiment 2, heifers (n=22) were treated similarly, except that 5.5 d after wave emergence, half the heifers in each group were further divided to be given 0.25 mg estradiol benzoate im or nothing, and 8 h later, all heifers were given 100 µg GnRH. The two

groups that were given E_2 (Low-and High-P_4) and the Low-P_4 group without E_2 had higher peak plasma LH concentrations ($P<0.04$) compared to the group with high P_4 without E_2 (12.6±1.8, 10.4±1.8, 8.7±1.3, and 3.9±1.2 ng/mL). However, E_2 did not affect ovulation rates ($P=0.6$). Combined for both experiments, heifers with low plasma P_4 concentrations had higher LH concentrations following GnRH treatment (11.5 vs 6.4 ng/mL, $P=0.039$). In conclusion, the hypotheses that higher doses of GnRH will be more efficacious in inducing LH release and that exogenous estradiol will increase LH release following treatment with GnRH were supported. Moreover, high concentrations of P_4 decreased both plasma LH concentrations and ovulation rates.

Key-words: Estradiol; Progesterone; GnRH; LH; Ovulation. Cattle

1. Introduction

Gonadotrophin-releasing hormone (GnRH) is a decapeptide synthesized and released by the hypothalamus, which acts on the anterior pituitary to induce the release of FSH and LH [1]. Steroid hormones regulate gonadotrophin release by both positive or negative feedback on the hypothalamus and/or anterior pituitary [2, 3]. Progesterone (P_4) suppresses LH release, whereas estradiol (E_2) enhances LH surges [2, 3]. The response of pituitary cells in culture to GnRH was associated with the number of GnRH receptors on the surface of the gonadotropes [4]. Increased peripheral P_4 concentrations decreased the frequency of GnRH pulses secreted by the hypothalamus and the number of GnRH receptors in the anterior pituitary [2, 5]. In this regard, we recently reported that high plasma P_4 concentrations suppressed pituitary release of LH and reduced the ovulatory response following the administration of 100 μg GnRH in both beef heifers and cows [6].

Pituitary LH release was increased with greater doses of GnRH in anestrous, diestrous, and ovariectomized ewes [7] and in ovariectomized cows [8]. Although the magnitude of plasma LH response increased with increasing doses of GnRH in diestrous heifers, the greatest increase occurred between 50 and 100 μg [9], suggesting that P_4 concentrations may influence the pituitary response to GnRH doses exceeding 100 μg. However, Mihm et al. [10] reported that 250 μg GnRH was more effective than 100 μg GnRH [MISTAKE: you have 250 ug in both places; Marcos: I changed it] to induce ovulation in diestrous beef heifers. Hence, whether the pituitary response of ovary-intact cattle to various doses of GnRH can be modulated by peripheral P_4 concentrations remains to be investigated.

In contrast to the effects of P_4, E_2 increases pituitary responsiveness to GnRH by increasing the expression of the gene encoding the GnRH receptor [3]. Furthermore, exposure of pituitary cells to E_2 for 24 h increased the secretion of LH into the incubation medium three-fold [11], indicating that E_2 can act directly on the hypophisis. However, E_2 treatment during the ovine luteal phase failed to increase the number of GnRH receptors on pituitary cells [12]. Hence, the role of E_2 in amplifying LH secretion in P_4-treated animals is not well established.

The objectives of the present study were to determine whether LH release and ovulation in response to GnRH treatment in heifers is influenced by peripheral P_4 concentrations, and whether P_4-induced suppression of LH release can be overcome by an increased dose of exogenous GnRH or pretreatment with E_2. We hypothesized that higher doses of GnRH or pretreatment with exogenous E_2 will enhance LH release and ovulatory response to exogenous GnRH in cattle with elevated peripheral P_4 concentrations.

DRAFT (Confidential)

2. Materials and methods

2.1. Cattle

Two experiments (2 x 2 factorial designs) were conducted using nulliparous crossbred Angus heifers (14-18 mo) in which a corpus luteum (CL) had been previously detected by transrectal ultrasonography. Heifers were maintained in outdoor pens at the University of Saskatchewan Goodale Research Farm (52° North and 106° West). All procedures were performed in accordance with the Canadian Council on Animal Care and were approved by the University of Saskatchewan Protocol Review Committee.

2.2. Experiment 1

Heifers (n=32) at random stages of the estrous cycle were given two luteolytic doses of 500 µg cloprostenol im (Estrumate, Schering-Plough Animal Health, Pointe-Claire, QC, Canada) 13 d apart, and assigned randomly to 4 treatment groups (n=8 per group) in a 2 x 2 factorial design to determine the effect of progesterone concentration (high vs low) and the dose of GnRH (100 vs 200 µg) on ovulatory response. Starting 4 d after the second cloprostenol treatment, heifers were examined daily by transrectal ultrasonography, using a 7.5 MHz linear-array transducer (Aloka SSD-900; Tokyo, Japan) to detect ovulation. Five days after ovulation, the two largest ovarian follicles were ablated by transvaginal ultrasound-guided follicle aspiration (CITE REFERENCE; Marcos: we could cite [13] here) to induce emergence of a new ovarian follicular wave (expected 1.5 d later [13]). Heifers were given a once-used intravaginal P_4-releasing insert (CIDR; Pfizer Canada Inc., Montreal, QC, Canada) and equal numbers were randomly assigned to be given two treatments of 25 mg dinoprost (PGF; Lutalyse; Pfizer Canada Inc.) im, 12 h apart (Low-P_4 group; n=16), or no treatment (High-P_4 group; n=16); i.e. heifers in the High-P_4 group were allowed to retain their CL. The Low-and High-P_4 groups were expected to have sub-physiological (i.e. <2.5 ng/mL [14]) and typical luteal phase plasma P_4 concentrations, respectively, during dominant follicle development. Six days after follicle wave emergence, half of the heifers in each group were given either 100 µg gonadorelin (GnRH; Cystorelin, Merial Canada Inc., Victoriaville, QC, Canada) or 200 µg of GnRH. To detect ovulation, heifers were examined by transrectal ultrasonography once daily for 4 d after GnRH treatment. The CIDR inserts were removed either on the day of ovulation, or 4 d after GnRH treatment in heifers which failed to ovulate.

Blood samples were collected by coccygeal venipuncture into 10 mL heparinized, evacuated tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ, USA) at CIDR insertion and at 0, 1, 2, 3, and 4 h after GnRH treatment. Samples were centrifuged at 1500 x g for 15 min, and plasma was separated and stored at -20 °C until assayed for P_4, E_2, and LH.

2.3. Experiment 2

DRAFT (Confidential)

Similar to Experiment 1, heifers (n=22) were treated with two doses of cloprostenol im, 13 d apart, and assigned randomly to 4 treatment groups in a 2 x 2 factorial design to determine the effect of P_4 concentration (high vs low) and estradiol treatment (yes vs no) on ovulatory response. To detect ovulation, heifers were examined daily by transrectal ultrasonography for 4 d after the second cloprostenol treatment. Follicular ablations were performed 5 d after ovulation, as in Experiment 1. Heifers were given a once-used CIDR and half (n=11) were given two doses of $PGF_{2\alpha}$ 12 h apart (Low-P_4 group) starting at ablation, whereas the remaining heifers were not given $PGF_{2\alpha}$ (High-P_4 group); heifers in the latter group were allowed to retain their CL. On Day 5.5 (wave emergence = Day 0), six heifers in each progesterone group were given 0.25 mg im estradiol benzoate (Sigma Chemical Co., St. Louis, MO, USA, prepared in in 0.2 ml canola oil), and 8 h later (i.e., Day 6), all heifers were given 100 µg GnRH. Heifers were examined by transrectal ultrasonography, once daily, for 4 d after GnRH treatment (to detect ovulation). The CIDR inserts were removed on the day of ovulation, or 4 d after GnRH treatment in heifers that failed to ovulate. Blood samples were collected, processed, and subsequently assayed, as described in Experiment 1.

2.4. Radioimmunoassays

Plasma LH concentrations were measured in duplicate using a double-antibody radioimmunoassay (NIDDK-bLH4) [15]. The range of the standard curve was 0.06 to 8.0 ng/mL. In Experiment 1, the intra-assay coefficients of variation were 8.2 and 9.5% for the low and high reference plasma (means, 1.1 and 2.7 ng/mL, respectively). In Experiment 2, the intra-assay coefficients of variation were 3.1 and 5.6% for the low and high reference plasma (means, 1.1 and 2.4 ng/mL) and the inter-assay coefficients of variation were 7.3 and 9.8%.

Plasma P_4 concentrations were measured using commercial solid-phase kits (Coat-A-Count; Diagnostic Products Corporation, Los Angeles, CA, USA). The range of the standard curve was 0.1 to 40.0 ng/mL. In Experiment 1, the intra-assay coefficients of variation were 9.7, 4.8, and 8.3% for low-, medium- and high-reference plasma (means, 1.9, 3.8 and 17.2 ng/mL). In Experiment 2, the intra-assay coefficients of variation were 4.7, 2.1 and 2.5% for the low-, medium- and high-reference plasma (means, 1.8, 3.5, and 16.5 ng/mL), whereas the inter-assay coefficients of variation were 9.0, 6.0, and 7.0%.

Plasma E_2 concentrations were measured using a modified commercial RIA kit designed for human samples (DPC Coat-a-Count; Diagnostic Products Corporation). In Experiment 1, the intra-assay coefficients of variation were 5.9 and 7.9% for the low- and high-reference plasma (means, 13.8 and 61.6 ng/mL). In Experiment 2, the intra-assay coefficients of variation were 8.9 and 4.0% for the low and high reference plasma (means, 13.8 and 53.4 ng/mL) and the inter-assay coefficients of variation were 6.7 and 9.8%.

2.6. Statistical analyses

For both experiments, statistical analyses were performed using the Statistical Analysis System software package (SAS Learning Edition 4.1, 2006; SAS Institute Inc., Cary, NC, USA). Where appropriate, continuous data were analysed as a 2 x 2 factorial. Time-series hormone data

DRAFT Confidential

were analyzed by ANOVA for repeated measures, using the MIXED procedure. For Experiment 1, the analysis included main effects of P_4 concentration (low vs high), GnRH dose (low vs high), and time. Similarly, for Experiment 2, main effects were P_4 concentrations (low vs high), estradiol (yes vs no), and time.

Two-way ANOVA were used to compare follicle diameter and plasma concentrations of progesterone and estradiol at the time of CIDR insertion and GnRH treatment. Ovulation rates were analyzed by Fisher's exact test.

Data from both experiments were combined to evaluate LH concentrations following treatment with 100 µg of GnRH in heifers with low versus high plasma progesterone concentrations. The analysis (repeated-measures, MIXED procedure) included only heifers that were given 100 µg of GnRH and no estradiol.

All values are expressed as mean ± SEM. If the main effects or interactions were significant ($P \leq 0.05$), Tukey's post-hoc test for multiple comparisons was used to locate differences.

3. Results

3.1. Experiment 1

Plasma P_4 concentrations were not significantly different between groups at CIDR insertion, but were lower in the Low-P_4 groups than in the High-P_4 groups at GnRH treatment ($P<0.001$; Table 1). The diameter of the largest ovarian follicle ($P=0.001$) and plasma E_2 concentration ($P=0.005$) at GnRH treatment were higher in the Low-P_4 groups than in the High-P4 groups (P=0.001 and 0.005, respectively). The diameter of the largest follicle prior to ovulation tended to be greater in the Low-P_4 groups ($P=0.09$). Fifteen of 16 heifers ovulated in the Low-P_4 groups, whereas only 6 of 16 heifers ovulated in the High-P_4 groups ($P=0.002$). Overall, the dose of GnRH did not have a significant effect on ovulation rates (9/16, 56% vs 12/16, 75% heifers given 100 vs 200 µg of GnRH, respectively; $P=0.4$). However, among heifers that received high progesterone, 1/8 vs 5/8 ovulated in response to treatment with 100 vs 200 µg of GnRH, respectively ($P=0.1$ for 2-sided Fisher's Exact test).

The LH response to GnRH treatment was affected significantly by P_4 concentration and GnRH dose (Fig. 1). Plasma LH concentrations were higher in the Low-P_4 vs High-P_4 group, and higher in the 200 vs 100 µg GnRH group. Overall, LH concentrations were higher at 1 and 2 h after GnRH treatment, than at 0, 3, or 4 h.

3.2. Experiment 2

As in Experiment 1, plasma P_4 concentrations were similar between groups at the time of CIDR insertion, but were lower in the Low-P_4 groups than in the High-P_4 groups at the time of GnRH treatment ($P<0.001$; Table 2). Plasma E_2 concentrations were elevated at GnRH treatment in the Low-P_4 groups ($P=0.02$) and groups pre-treated with E_2 ($P<0.001$). The diameter of the

DRAFT confidential

largest follicle on the day of GnRH treatment and on the day prior to ovulation was greater ($P<0.01$) in the Low- vs High-P_4 groups; there was no main effect or interaction with estradiol treatment. All heifers (11/11) ovulated in the low-P_4 groups, whereas only 7/11 heifers ovulated in the High-P_4 groups ($P=0.09$). Irrespective of P_4 concentration, E_2 treatment prior to GnRH did not influence ovulation rate (9/12, 75% vs 9/10, 90% heifers ovulated with vs without E_2, $P=0.6$).

The LH response to GnRH treatment was affected significantly by P_4 concentration and estradiol treatment (Fig. 2). Plasma LH concentrations were higher in the Low- vs High-P_4 groups, and overall, LH concentrations were higher at 1 and 2 h after GnRH, than at 0, 3, or 4 h.

3.3. Experiments 1 and 2 combined

The post-GnRH treatment surge in plasma LH concentration was attenuated in heifers with high P_4 concentrations compared to those with low P_4 (time x P_4 interaction, $P=0.01$; Fig. 3).

5. Discussion

The hypotheses that higher doses of GnRH will be more efficacious in inducing LH release and that exogenous E_2 will increase LH release following the administration of GnRH were supported. Moreover, high P_4 concentrations suppressed both the LH and ovulatory responses to GnRH treatment, and heifers in the High-P_4 groups had lower plasma E_2 concentrations prior to ovulation in both experiments. Marcos: This confirms our findings in paper [6]. As expected, plasma P_4 concentrations did not differ between groups prior to CIDR treatment in both experiments, and were significantly lower in the Low-P_4 group at the time of GnRH treatment. A new follicular wave emerged approximately 1 d after follicular ablation in both the high- and low-P_4 groups, consistent with a previous report that this procedure increased plasma FSH concentrations and resulted in wave emergence approximately 1.5 d after follicle ablation [13]. Therefore, treatments to induce different P_4 environments and to synchronize emergence of follicular waves were highly successful.

In the present study, high plasma P_4 concentrations resulted in a smaller dominant follicle at GnRH treatment and prior to ovulation, decreased serum E_2 concentrations, and reduced ovulation rates. Similarly, it was previously reported that elevated P_4 concentrations 3 d prior to ovulation resulted in a smaller diameter follicle [16, 17], reduced E_2 concentrations, and decreased fertility [18] . The effect of P_4 on follicle diameter was likely due to suppression of LH secretion; once a dominant follicle emerges, its continued growth is largely dependent on LH [19, 20]. The suppression of plasma E_2 concentrations by P_4 has been previously reported and was probably associated with suppressed gonadotrophin secretion [21].

Exogenous estradiol benzoate given 8 h before GnRH mimicked the natural increase in E_2 that occurs prior to the onset of estrus [22] Marcos: This is confusing; if you refer to reference 22 they used E-17B after PGF treatment, and enhanced LH release in heifers with both low and high P_4 concentrations. In particular, E_2 treatment of heifers with high P_4 significantly increased LH release. However, despite higher LH concentrations, ovulation rate was not increased in the present study.

DRAFT (Confidential)

Regardless of progesterone concentration, ovulation rates were not affected by treatments in both experiments (i.e. GnRH dose for Experiment 1 and pre-treatment with estradiol forEexperiment 2). The limited number of heifers per group in both experiments limited statistical power for the effect of treatments (GnRH dose or E_2) on ovulation. Regardless, GnRH dose in high-P_4 groups tended to affect ovulation rates. An experiment utilizing more animals is needed to confirm these trends.

When LH data were combined for the two experiments, the effects of P_4 concentrations on LH release and ovulation rates were more obvious. Lower concentrations of P_4 during growth of the ovulatory follicle has been shown to increase LH pulse frequency, follicle size and ovulation rates [6, 23, 24]. Dominant follicles in cattle acquire LH receptors at a diameter of approximately 8.5 mm [18, 25], and LH secretion is the impetus for dominant follicle growth. However, when the dominant follicle developed in a high P_4 environment, its diameter was smaller [14] and it presumably required a higher LH peak to ovulate [6]. In the present study, a small dose of estradiol benzoate 8 h before GnRH treatment increased LH release, but was not sufficient to increase ovulation rate.

In conclusion, physiological luteal-phase concentrations of plasma P_4 during the growth of a dominant follicle in cattle suppressed the GnRH-induced release of LH and decreased ovulation rate compared with sub-luteal phase P_4 concentrations. The suppressive effect of P_4 on LH release was overcome in part by increasing the dose of GnRH from 100 to 200 μg. Estradiol benzoate treatment prior to administration of GnRH promoted LH release in heifers with elevated plasma P_4 concentrations, but did not significantly increase ovulation rate. For the purposes of ovulation synchronization, the LH and ovulatory responses to GnRH treatment may be increased by minimizing circulating P_4 concentrations at treatment. Furthermore, the suppressive effect of P_4 on GnRH response may be mitigated, at least in part, by increasing the dose of GnRH or by pretreatment with estradiol benzoate.

Acknowledgements

The project was supported by research grants from Saskatchewan Agriculture Development Fund, Westgen Inc. and the Natural Sciences and Engineering Research Council of Canada. The authors thank Pfizer Animal Health for CIDR inserts and Lutalyse, Schering-Plough Animal Health for Estrumate, and Merial Canada for Cystorelin. The authors acknowledge the help provided by Dr Marcelo Martinez for conducting Experiment 2 and thank personnel from the Goodale farm for providing cattle and technical assistance.

DRAFT (Original Copy)

6. References

1. Kaltenbach CC, Dunn TG, Kiser TE, Corah LR, Akbar AM, Niswender GD. Release of FSH and LH in beef heifers by synthetic gonadotrophin releasing hormone. J Anim Sci 1974; 38: 357-362.
2. Karsch FJ. Central actions of ovarian steroids in the feedback regulation of pulsatile secretion of luteinizing hormone. Annu Rev Physiol 1987; 49: 365-382.
3. Nett TM, Turzillo AM, Baratta M, Rispoli LA. Pituitary effects of steroid hormones on secretion of follicle-stimulating hormone and luteinizing hormone. Domest Anim Endocrinol 2002; 23: 33-42.
4. Wise ME, Nieman D, Stewart J, Nett TM. Effect of number of receptors for gonadotropin-releasing hormone on the release of luteinizing hormone. Biol Reprod 1984; 31: 1007-1013.
5. Turzillo AM, Juengel JL, Nett TM. Pulsatile gonadotropin-releasing hormone (GnRH) increases concentrations of GnRH receptor messenger ribonucleic acid and numbers of GnRH receptors during luteolysis in the ewe. Biol Reprod 1995; 53: 418-423.
6. Colazo MG, Kastelic JP, Davis H, Rutledge MD, Martinez MF, Small JA, Mapletoft RJ. Effects of plasma progesterone concentrations on LH release and ovulation in beef cattle given GnRH. Domest Anim Endocrinol 2008; 34: 109-116.
7. Rippel RH, Moyer RH, Johnson ES, Mauer RE. Response of the ewe to synthetic gonadotropin releasing hormone. J Anim Sci 1974; 38: 605-612.
8. Colazo MG, Ree TO, Emmanuel DGV, Ambrose DJ. Plasma luteinizing hormone concentration in cows given repeated treatments of various doses of gonadotropin releasing hormone. Theriogenology 2008; (in press).
9. Jensen AK, Pedersen KM, Agger JF, Madej A. Plasma luteinizing hormone response to increasing doses of synthetic gonadotrophin-releasing hormone in heifers. Acta Vet Scand 1983; 24: 211-224.
10. Mihm M, Delatang F, Roche JF. The gonadotrophin and ovarian responses to an intermediate or low dose of gonadorelin in beef heifers:influence of dose, follicle status and progesterone environment. J Reprod Fertil 1998; Abstract series 21: 74.
11. Baratta M, West LA, Turzillo AM, Nett TM. Activin modulates differential effects of estradiol on synthesis and secretion of follicle-stimulating hormone in ovine pituitary cells. Biol Reprod 2001; 64: 714-719.
12. Turzillo AM, Clapper JA, Moss GE, Nett TM. Regulation of ovine GnRH receptor gene expression by progesterone and oestradiol. J Reprod Fertil 1998; 113: 251-256.
13. Bergfelt DR, Lightfoot KC, Adams GP. Ovarian synchronization following ultrasound-guided follicular ablation in heifers. Theriogenology 1994; 42: 895-907.
14. Adams GP, Matteri RL, Ginther OJ. Effect of progesterone on ovarian follicles, emergence of follicular waves and circulating follicle-stimulating hormone in heifers. J Reprod Fertil 1992; 96: 627-640.
15. Evans AC, O'Keeffe P, Mihm M, Roche JF, Macmillan KL, Boland MP. Effect of oestradiol benzoate given after prostaglandin at two stages of follicle wave development on oestrus synchronisation, the LH surge and ovulation in heifers. Anim Reprod Sci 2003; 76: 13-23.

Confidential

16. Carvalho JB, Carvalho NA, Reis EL, Nichi M, Souza AH, Baruselli PS. Effect of early luteolysis in progesterone-based timed AI protocols in Bos indicus, Bos indicus x Bos taurus, and Bos taurus heifers. Theriogenology 2008; 69: 167-175.
17. Sartori R, Haughian JM, Shaver RD, Rosa GJ, Wiltbank MC. Comparison of ovarian function and circulating steroids in estrous cycles of Holstein heifers and lactating cows. J Dairy Sci 2004; 87: 905-920.
18. Ginther OJ, Bergfelt DR, Beg MA, Kot K. Follicle selection in cattle: role of luteinizing hormone. Biol Reprod 2001; 64: 197-205.
19. Mihm M, Crowe MA, Knight PG, Austin EJ. Follicle wave growth in cattle. Reprod Domest Anim 2002; 37: 191-200.
20. Ginther OJ, Bergfelt DR, Kulick LJ, Kot K. Selection of the dominant follicle in cattle: role of estradiol. Biol Reprod 2000; 63: 383-389.
21. Glencross RG. Effect of pulsatile infusion of gonadotropin-releasing hormone on plasma oestradiol-17 beta concentrations and follicular development during naturally and artificially maintained high levels of plasma progesterone in heifers. J Endocrinol 1987; 112: 77-85.
22. Souza AH, Gumen A, Silva EP, Cunha AP, Guenther JN, Peters CM, Caraviello DZ, Wiltbank MC. Supplementation with estradiol-17beta before the last gonadotropin-releasing hormone injection of the Ovsynch protocol in lactating dairy cows. J Dairy Sci 2007; 90: 4623-4634.
23. Adams GP, Matteri RL, Kastelic JP, Ko JC, Ginther OJ. Association between surges of follicle-stimulating hormone and the emergence of follicular waves in heifers. Journal of reproduction and fertility 1992; 94: 177-188.
24. Stock AE, Fortune JE. Ovarian follicular dominance in cattle: relationship between prolonged growth of the ovulatory follicle and endocrine parameters. Endocrinology 1993; 132: 1108-1114.
25. Ginther OJ, Beg MA, Bergfelt DR, Donadeu FX, Kot K. Follicle selection in monovular species. Biol Reprod 2001; 65: 638-647.

DRAFT (Confidential)

366 Table 1. The ovarian and hormonal response (mean ± SEM) in heifers with low (Low-P_4)
367 versus high (High-P_4) plasma progesterone (P_4) concentrations, treated with either 100 or
368 200 µg of GnRH to induce ovulation (Experiment 1).

	Low-P_4		High-P_4		Probability		
End point	100 µg GnRH	200 µg GnRH	100 µg GnRH	200 µg GnRH	P_4	GnRH	P_4*Gn
No. heifers	8	8	8	8	-	-	-
P_4 at CIDR insertion (ng/mL)	3.3 ± 0.3	2.8 ± 0.3	3.9 ± 0.9	3.1 ± 0.5	0.4	0.3	0.9
P_4 at GnRH treatment (ng/mL)	1.9 ± 0.4	2.3 ± 0.3	6.8 ± 0.4	6.9 ± 0.8	<0.001	0.6	0.8
E_2 at GnRH treatment (pg/mL)	11.3 ± 1.0	11.0 ± 1.3	5.2 ± 1.1	7.4 ± 1.4	0.005	0.4	0.3
Diameter of largest follicle at GnRH treatment (mm)	10.7 ± 0.4	11.7 ± 0.7	8.5 ± 0.3	9.1 ± 1.0	0.001	0.2	0.8
Diameter of largest follicle before ovulation (mm)	11.1 ± 0.3	12.7 ± 0.6	10 ± 0.0*	10.6 ± 0.9	0.09	0.25	0.6
Interval from GnRH treatment to ovulation (d)	1.8 ± 0.1	2.0 ± 0.0	2.0 ± 0.0*	1.8 ± 0.2	0.8	0.8	0.4

369 *Data from only one heifer.
370

DRAFT (Confidential)

370
371 Table 2. Ovarian and hormonal responses (mean ± SEM) in heifers with low (low-P_4)
372 versus high (high-P_4) plasma progesterone (P_4) concentrations, treated with or without estradiol
373 benzoate (E_2) 8 h before being given an ovulatory dose of GnRH (Experiment 2).

	Low-P_4		High-P_4		Probability		
Endpoint	No E_2	E_2	No E_2	E_2	P_4	E_2	P4*E_2
No. heifers	5	6	5	6	-	-	-
P_4 at CIDR insertion (ng/mL)	4.7 ± 0.8	5.6 ± 0.7	4.6 ± 0.8	4.0 ± 0.7	0.3	0.9	0.3
P_4 at GnRH treatment (ng/mL)	1.9 ± 0.9	1.7 ± 0.8	9.1 ± 0.9	8.3 ± 0.8	0.001	0.6	0.7
Estradiol at GnRH treatment (pg/mL)	11.4 ± 2.2	31.1 ± 3.1	7.1 ± 2.0	20.2 ± 4.4	0.02	<0.001	0.3
Diameter of largest follicle at GnRH treatment (mm)	12.9 ± 0.7	13.7 ± 0.6	10.3 ± 0.7	11.1 ± 0.6	0.009	0.2	0.9
Diameter of largest follicle before ovulation (mm)	14.0 ± 0.7	14.4 ± 0.7	10.8 ± 0.6	10.6 ± 0.6	0.001	0.9	0.6
Interval from GnRH treatment to ovulation (d)	1.4 ± 0.4	1.3 ± 0.4	0.6 ± 0.4	1.8 ± 0.4	0.7	0.2	0.1

374
375

DRAFT (Confidential)

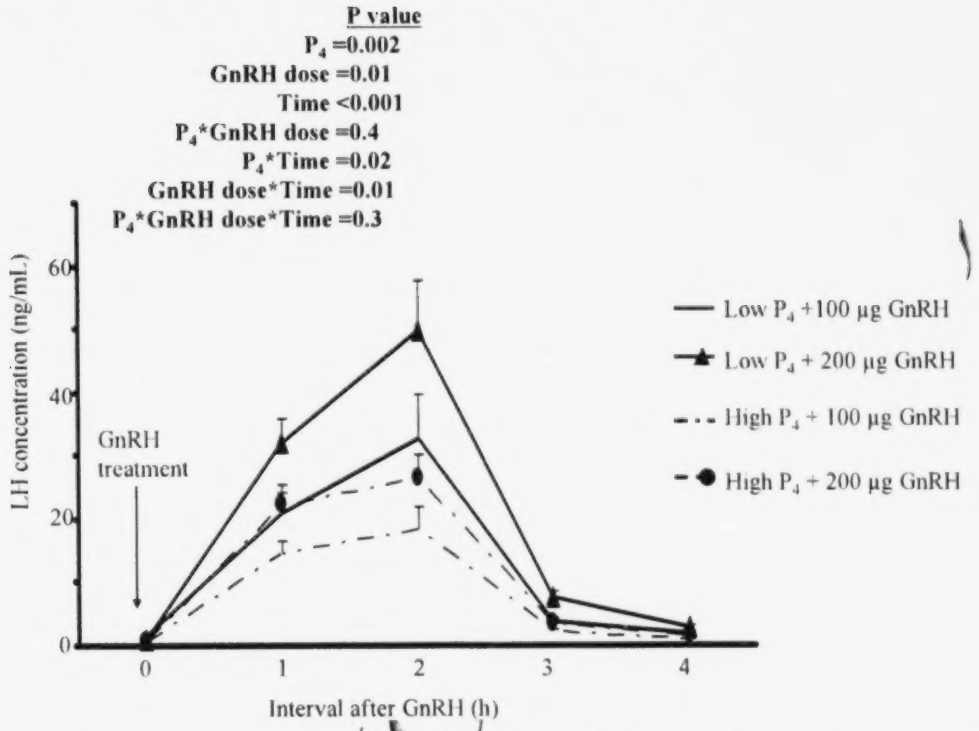
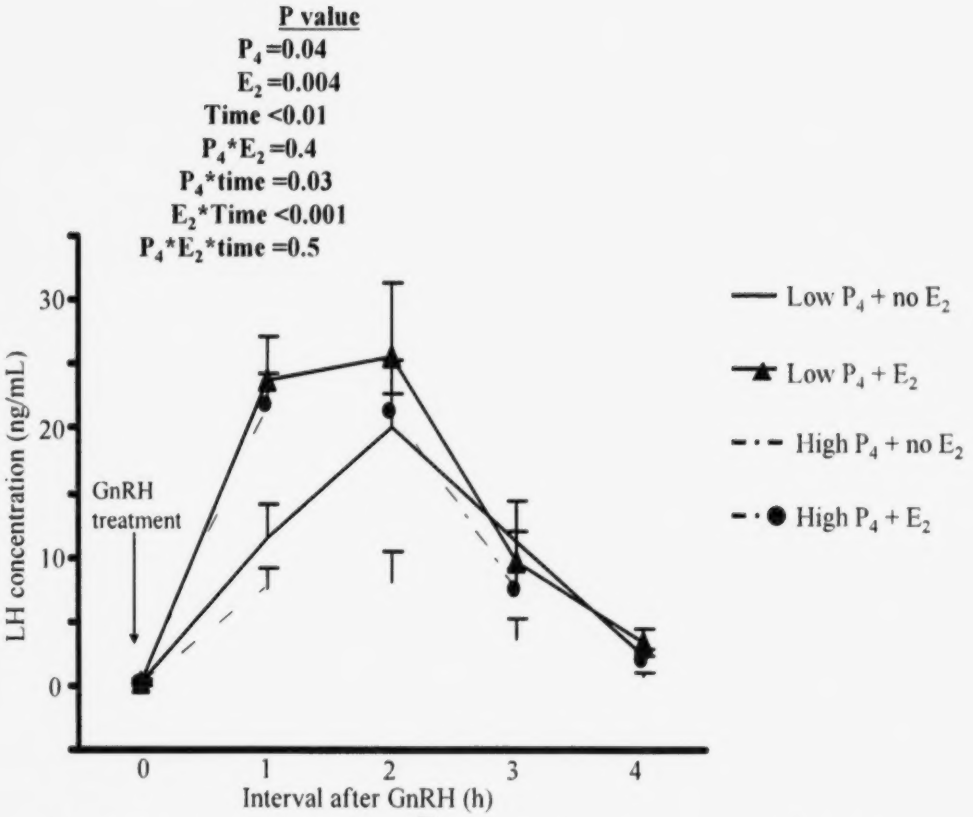


Fig. 1 Mean (±SEM) plasma concentrations of LH in heifers with low versus high plasma concentrations of progesterone (P_4), treated with either 100 or 200 µg GnRH to induce ovulation (Experiment 1; n=8 per group).

DRAFT

382



383

384 Fig. 2 Plasma concentrations (mean ±SEM) of LH in response to 100 GnRH μg treatment
385 in heifers with low versus high plasma concentrations of progesterone (P_4) and either pre-treated
386 or not pre-treated with estradiol benzoate (2.5 mg im 8 h before GnRH; Experiment 2).
387

DRAFT

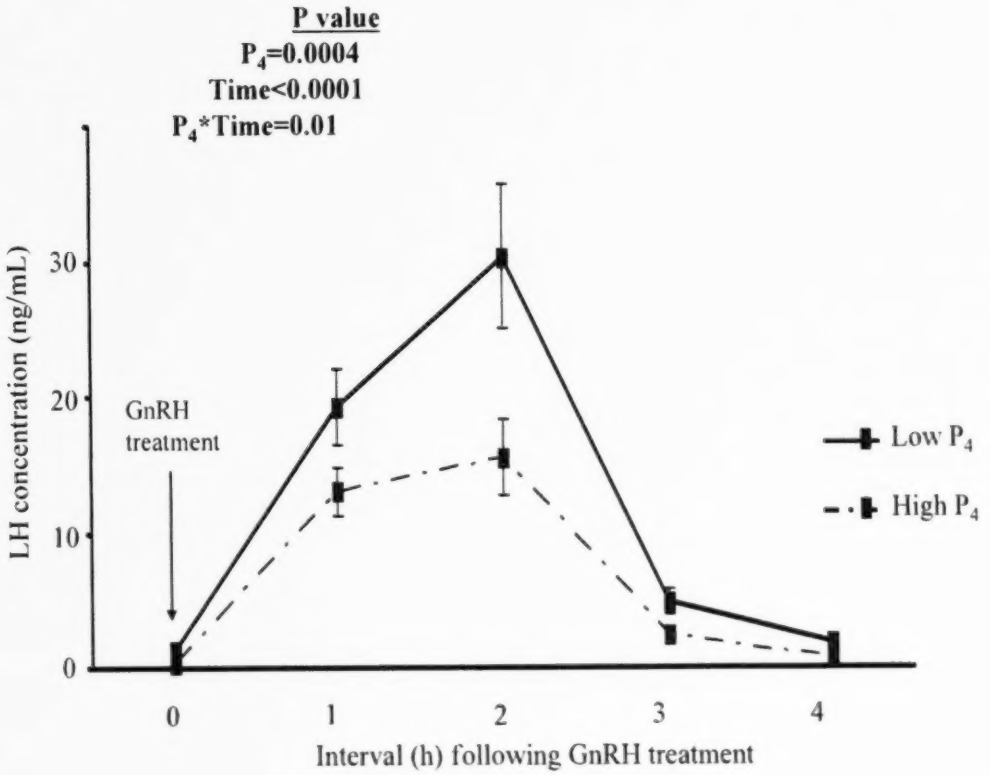


Fig. 3 Mean (±SEM) plasma concentrations of LH in response to treatment with 100 μg GnRH in heifers with low versus high plasma concentrations of progesterone (P_4; n=11 per group, combined for Experiments 1 and 2).

DRAFT